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Mini-review

Some molecular targets for antihyperlipidemic drug research

Nikhilesh Arya [a, b], Mangesh D. Kharjul [c], Chamanlal J. Shishoo [d], Vishnu N. Thakare [b], Kishor S. Jain [b, *]

[a] Department of Chemistry, Banasthali University, Tonk 304022, Rajasthan, India
[b] Department of Pharmaceutical Chemistry, Sinhgad Institute of Pharmaceutical Sciences, Lonavala, Pune 410401, Maharashtra, India
[c] Department of Pharmacology, S.S. Jondhale College of Pharmacy, Asangaon, Thane 421601, Maharashtra, India
[d] B.V. Patel Pharmaceutical Education and Research Development (PERD) Centre, S.G. Highway, Thaltej, Ahmedabad 380 054, Gujarat, India

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ABSTRACT

High levels of cholesterol and other lipid constituents are major risk factors in the development of atherosclerosis as well as diseases and disorders associated with it. Though, drugs of various categories acting through different mechanisms are available for antihyperlipidemic therapy, there are limitations associated with each of them, keeping the interest in discovery of newer and better antihyperlipidemic drugs alive. Identification and exploitation of novel molecular targets for discovery of new antihyperlipidemic drugs is an important area of research. Twenty such drug targets are elaborated herein, for their biochemical roles, structures, estimations, as well as, exploitation for new drug discovery research. Few recently discovered drugs are based on such molecular targets are also discussed.

1. Introduction

Cardiovascular diseases (CVD's) are among the major causes of deaths in the world today and shall continue to be so even by 2020 [1–3]. Hyperlipidaemia, leading to atherosclerosis is the major underlying factor for CVD's [1,4]. Elevated low-density lipoprotein cholesterol (LDLC) levels are the best indicators of the atherosclerotic risk [5]. Many novel molecular targets, on which new drugs could act and control hyperlipidaemia, are being identified and evaluated for new antihyperlipidemic drug discovery research [6].

The rising tide of obesity, diabetes and hypertension are collectively attributed to our reluctance to exercise and desire for fast food [7]. Atherosclerosis may be defined as degenerative changes in the intima of medium and large arteries. This degeneration includes accumulation of lipids, complex carbohydrates, blood and blood products, as well as, cellular debris leading to plaque formation [8]. As more plaques build up in the intima, arteries become narrow and stiffen. Eventually, enough plaques may build up to reduce blood flow through the arteries. This results in blocking a blood vessel or vessels that feed the heart, precipitating a heart attack. If plaques block blood vessels that feed the brain, it can cause a stroke. On the other hand if blood supply to the arms or legs is reduced, it can lead to gangrene or paralysis [9]. Treatment of the atherosclerotic conditions of cardiovascular systems with procedures such as coronary artery by-pass graft (CABG), insertion of stents as well as use of various pharmaceutical agents to treat hypertension, diabetes, dyslipidaemia, pose an enormous economic, as well as, social burden on the society.

Cessation of smoking, control of blood pressure and blood levels of glucose, low density lipoprotein cholesterol (LDLC), as well as, elevation of high density lipoprotein cholesterol (HDLC) levels remain the most effective long-term options for controlling atherosclerosis [6]. More emphasis has been focused on the management of cholesterol, primarily through lifestyle and drug therapy. Drug therapy offers numerous options, with each drug class dealing with the disease state through its own unique mechanism of action. In addition, different cholesterol lowering drugs or non-pharmacological treatments can significantly reduce morbidity from CVDs and the related coronary events.

Two different classifications for hyperlipidaemias are; the Fredrickson classification [10] and the WHO classification [11], both based on the levels of lipoproteins, triglycerides and chylomicron in the blood.

* Corresponding author. Department of Pharmaceutical Chemistry, Sinhgad Institute of Pharmaceutical Sciences, S. No. 309/310, Kusgaon (Bk.), Off. Mumbai–Pune Expressway, Lonavala, Pune 410 401, Maharashtra, India.
E-mail address: drkishorsjain@gmail.com (K.S. Jain).

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Lipoproteins are small spherules that transport fats in the body and consist of cholesterol, triglycerides and phospholipids.

Lipoproteins are classified as chylomicrons, very low-density lipoprotein (VLDL), intermediate density lipoprotein (IDL), LDL and HDL [12] based on their electrophoresis, density and composition [13–20], the HDL being the smallest, but most dense amongst various lipoproteins [12]. Apolipoproteins found on the outer surface of lipoprotein, make them soluble in plasma [21].

The terms “good” and “bad” cholesterol refer to HDL and LDL, respectively. High levels of LDL are associated with coronary atherosclerosis, whereas, high levels of HDL appear to protect against CVD's [22–26].

2. Pathways of lipid transport

Cholesterol is essential for the production of hormones and vitamins in the body, as well as, for the integrity of cell membranes. The lipid metabolism and transport involves exogenous and endogenous pathways in the body. Liver and intestine are the main organs involved in the lipid and lipoprotein metabolism [27]. Various enzymes namely, lipoprotein lipase [28], hepatic triglyceride lipase [29], lecithin cholesterol acyl transferase (LCAT) [30], cholesterol ester transfer protein (CETP) [31], microsomal triglyceride protein (MTP) [32] and acyl CoA cholesterol acyl transferase (ACAT) [33] are involved in these lipid metabolic processes.

In the exogenous pathway [34], chylomicrons transport dietary lipids that are absorbed from the intestine *via* the systemic circulation. In the endogenous pathway [35], the liver assembles and secretes triglyceride-rich VLDL particles, which transport triglycerides from liver to the peripheral tissues.

In reverse cholesterol transport (RCT) pathway cholesterol is transported from atherosclerotic plaques or other lipids back to the liver to be excreted into the feces through bile [35]. The cholesterol from cells and their turnovers are recovered and reincorporated into IDL pool or returned to the liver [36].

3. Antihyperlipidemic agents currently used in therapy

Antihyperlipidemic drugs are broadly classified into 5 main types (Table 1) [5].

Though drugs of various categories acting through different mechanisms are available for the management of hyperlipidaemia, there are a few limitations [37] associated with the anti-hyperlipidemic therapy as enlisted below;

a) Drugs like clofibrate, nicotinic acid, D-thyroxine etc., are not very effective therefore, new drugs are required for treatment of the hitherto untreatable cases of Type II hyperlipidaemia.
b) New drugs, able to block the stimuli responsible for the formation of an atherosclerotic lesion need to be developed.
c) Furthermore, specific drugs, which could bring about regression of the already existing atherosclerotic lesions, in the blood vessels are the need of the hour.
d) The most widely used “Statins” suffer from limitations like, intolerance and adverse effects, often achieving only 40% risk reduction and sometimes even ineffective.

Therefore, novel potential molecular targets for new drug discovery research (NDDR) for antihyperlipidemic therapy are being searched and investigated. Though, earlier this topic has been reviewed [37], the interest in antihyperlipidemic research, as well as, the constant development in the field, makes it necessary to have a periodic update on this subject.

This review covers in details the roles of these twenty different molecular targets in the biochemical and biosynthetic lipid pathways, literature reports on their recent studies till 2013, as well as, information on some molecules identified as their agonists/antagonists, to help design novel new chemical entities (NCE's). The X-ray crystal structures of many of these molecular targets are also dealt with and could be useful for molecular docking studies. Further, the biochemical assay procedures for the *in vitro* evaluation of potential leads against these targets have also been provided.

4. Current drug targets for antihyperlipidemic therapy

4.1. Molecular entities involved in absorption of cholesterol [inhibition of cholesterol absorption]

Identifying entities in the body responsible for absorption of the dietary cholesterol and blocking them specifically, is an area of

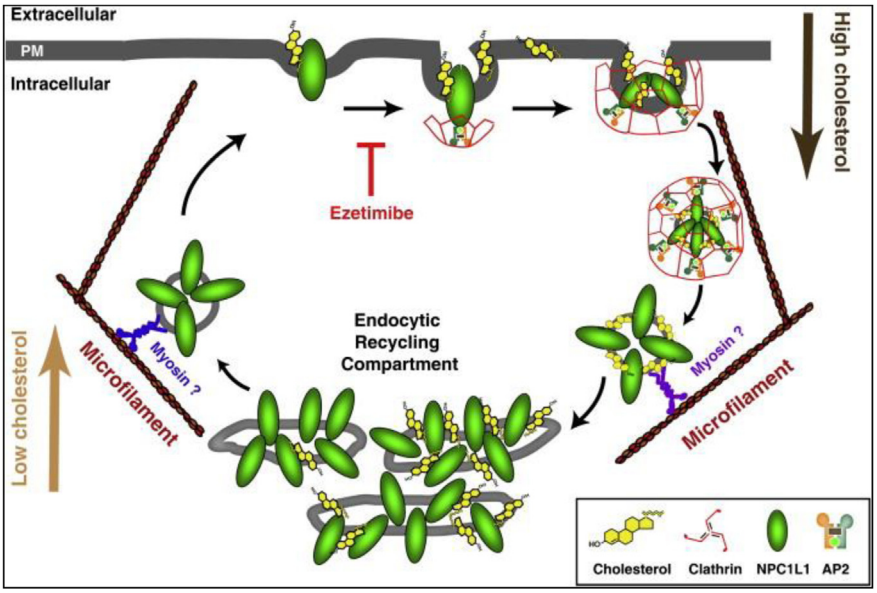

Fig. 1. NPC1L1 protein and ezetimibe as its blocker.

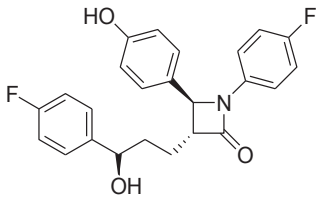

Fig. 2. Ezetimibe.

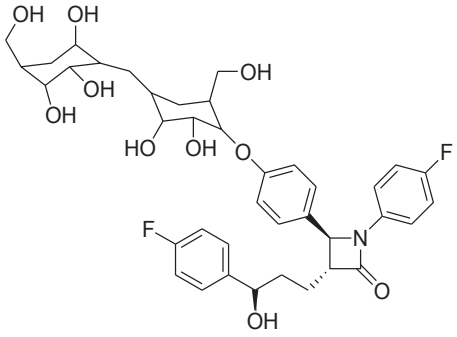

Fig. 3. Nonhydrolysable phenolic glycosides of ezetimibe.

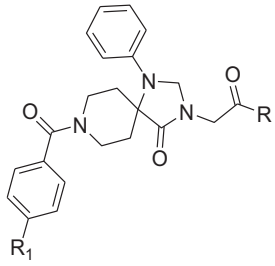

Fig. 4. Spiroimidazolidinone derivatives.

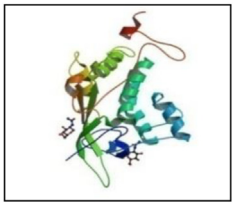

Fig. 5. Crystal structure of Niemann-Pick C1-like protein 1 (NPC1L1) – 3QNT.

interest in current antihyperlipidemic drug research. Such entities form attractive targets for the NDDR. One such entity is the NPC1L1 (Niemann–Pick C1-like-1) protein located at the apical membrane of intestinal enterocytes and facilitates the uptake of cholesterol across the brush border membrane (Fig. 1) [38]. Some studies are in progress to establish the existence of another new class of proteins, e.g., CUP-1, involved in sterol absorption in mammals including humans [39].

Indeed on this basis, NDDR has led to the discovery and development of the drug ezetimibe (Fig. 2), which acts through the selective inhibition of cholesterol absorption and blocking sterol transport, to reduce LDLC significantly. It is taken as monotherapy or synergistically with statins [40]. Attempts have been made to modify various positions of the basic scaffold 2-azetidinone [41]. Of interest are a few such examples that are discussed herein.

Substituents on the lactam ring have resulted in wide range of nonhydrolysable phenolic glycosides of ezetimibe (Fig. 3).

Promising cholesterol absorption inhibitory (CAI) activity has also been observed for non-β-lactams like, spiroimidazolidinone derivatives (Fig. 4) in a rodent model [42]. These compounds were evaluated in a binding assay of mouse brush border membranes and also in human embryonic kidney 293 cell line which expressed recombinant human NPC1L1. Good binding affinities to human NPC1L1 were observed.

By real time imaging of enterocyte lipid droplets in live zebrafish larvae, J.W. Walters et al. [43] have demonstrated rapid re-localization of NPC1L1 to intestinal brush border promoting intestinal cholesterol absorption. This was studied by inclusion of fluorescently labelled BODIPY-fatty acids in the high atherogenic lipid diet that was fed to live zebrafish larvae.

H.J. Kwon et al. [44] through the crystal structure of NPC1L1 have indicated that the cholesterol selectivity of NPC1L1 is due to its N-terminal domain (NTD), which exists in a closed conformation. The occlusion of sterol binding pocket suggests a gating mechanism (Fig. 5).

L.J. Wang et al. [45] have analysed 19 reported nonsynonymous (NS) variants of NPC1L1 in humans which are summarized in Table 2.

Flottilins (lipid raft scaffold proteins) associated with NPC1L1, form cholesterol enriched micro domains which, mediate cellular cholesterol uptake, biliary cholesterol reabsorption and regulation of lipid levels in mice. Ezetimibe disrupts this association between NPC1L1 and these flotillins to exert its hypocholesterolemic activity [46].

Y. Zhu et al. [47], have determined the serum levels of cholesterol, triglycerides and NPC1L1 as an index of cholesterol absorption in a rat orthotopic small bowel transplantation (OSBT) model, during the development of chronic rejection of the transplantation in it. They found that increased expression of NPC1L1 contributes to hypercholesteromia, leading to pathogenesis of transplant arteriosclerosis.

Glucose appears to be directly modulating the NPC1L1 expression *via* transcriptional mechanisms and involvement of phosphatase dependent pathways in human intestinal Caco-2 cells [48].

Transgenic overexpression of NPC1L1 in the wild-type mouse liver inhibits biliary cholesterol secretion and raises blood cholesterol, which can be reversed by ezetimibe treatment [49]. These findings have demonstrated a direct role of hepatic NPC1L1 in regulating biliary cholesterol excretion and hepatic/blood cholesterol levels and unequivocally established hepatic NPC1L1 as an important target for ezetimibe.

4.1.1. In vitro biological evaluation protocol for NPC1L1 inhibitory activity [50]

The underlying principle for this assay involves the radiolabelled competitive *in vitro* binding studies at NPC1L1 by the ligands (test compounds) *vis-à-vis* [^{3}H]ezetimibe-glucuronide (EZE-gluc).

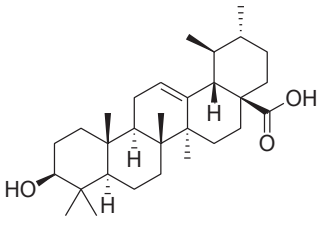

Fig. 6. Ursolic acid.

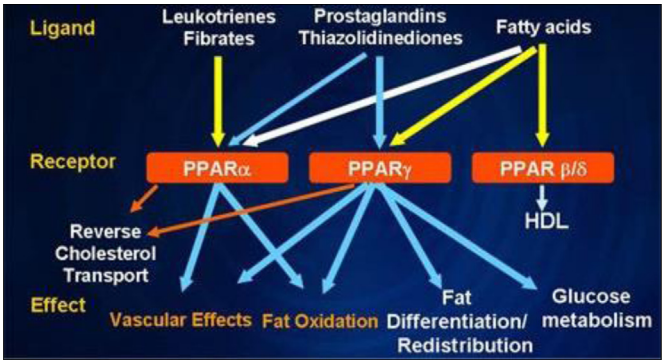

Fig. 7. Mechanisms of genetic regulation by PPARs.

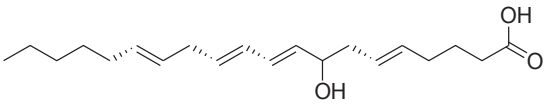

Fig. 8. 8-HETE.

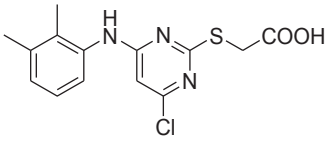

Fig. 9. Wy-14643.

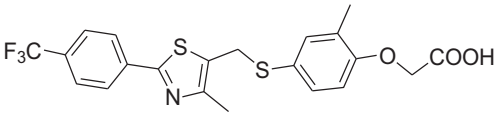

Fig. 10. GW-501516.

4.1.1.1. [^{3}H]Ezetimibe-glucuronide (EZE-gluc) binding assay. It is conducted in 12 × 75 mm glass test tubes with a total volume of 20–100 μl. Frozen mucosal scrapings of rat (male Sprague–Dawley) intestine are diluted in buffer A (Composition: 26 mM $NaHCO_3$/ 0.96 mM NaH_2PO_4/5 mM Hepes/5.5 mM glucose/117 mM NaCl/ 5.4 mM KCl, pH 7.40) alone or buffer A containing 0.03% taurocholate and 0.05% digitonin, to a final concentration of 0.5–5 mg/ml. Final concentrations of [^{3}H] EZE-gluc are typically 25–50 nM and are delivered as DMSO or CH_3CN solutions. Reaction mixture is incubated until equilibrium is achieved (up to 1 h). Bound ligand (test compounds) is recovered by single-tube vacuum filtration on GF/C glass fibre filters (Whatman). The filter is counted in 7 ml vials by using Ultima Gold MV liquid scintillation fluid (Packard). Triplicate assay is performed.

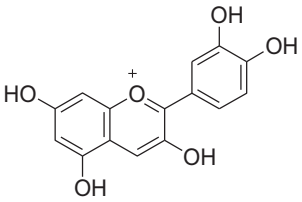

Fig. 12. Cyanidin.

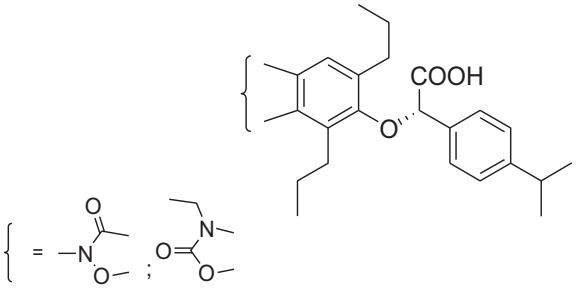

Fig. 13. α-Aryloxyphenylacetic acids.

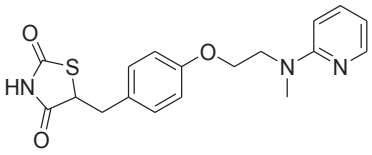

Fig. 14. Rosiglitazone.

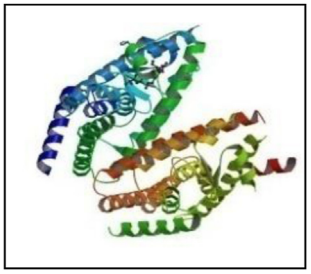

Fig. 15. Crystal structure of human PPAR-γ ligand binding domain complexed with an alpha-aryloxyphenylacetic acid against – 1ZEO.

4.2. Peroxisome proliferation activated receptors (PPARs) [agonists of PPARs]

Lipid metabolism, glucose metabolism, morphogenesis and cell homoeostasis are mainly regulated by ligand dependent nuclear transcription factors or receptors like PPARs [51]. In liver and other

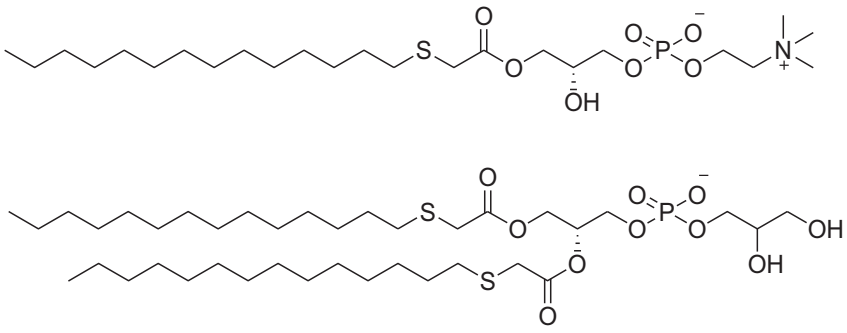

Fig. 11. Phospholipid analogues of tetradecylthioacetic acid (TTA).

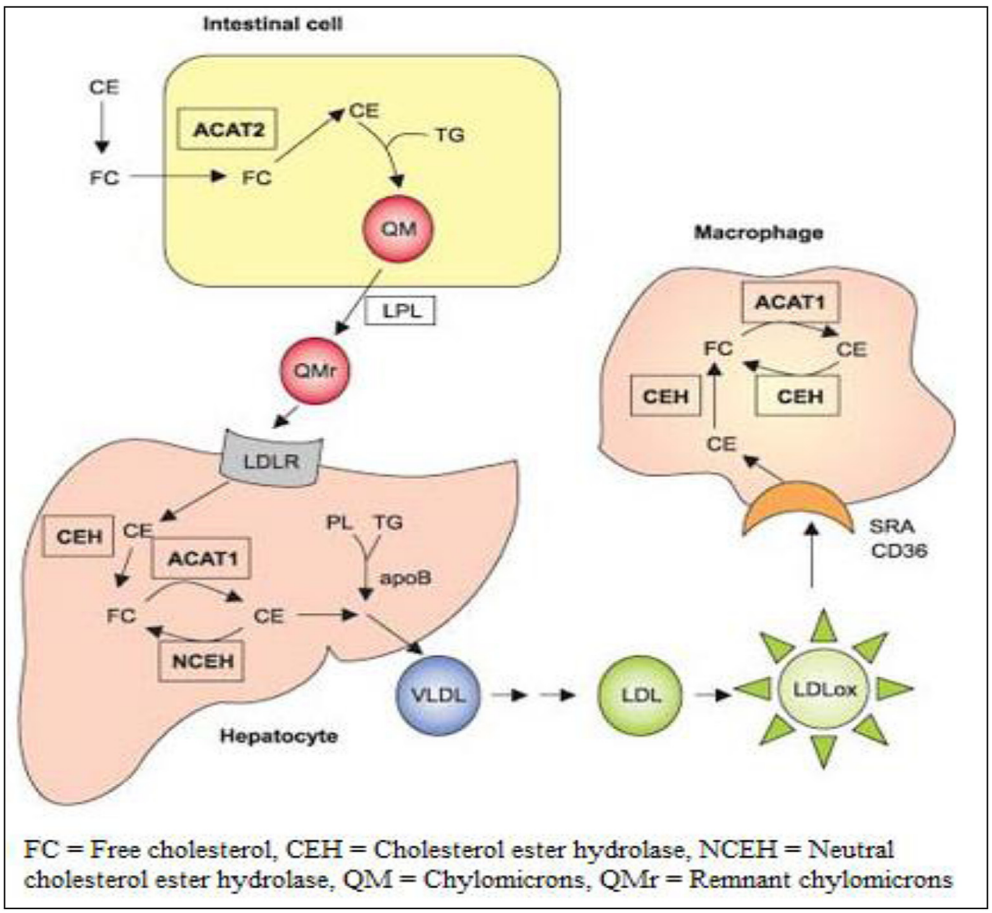

Fig. 16. Mechanisms of ACAT-1 and ACAT-2.

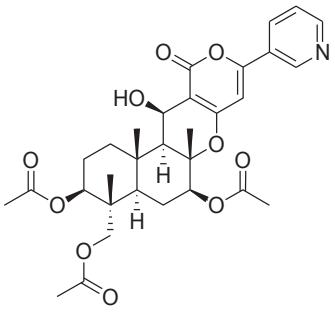

Fig. 17. Pyriproprene A.

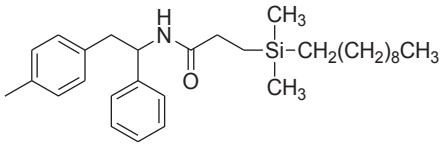

Fig. 19. Sandoz-58-035.

tissues, lipoprotein synthesis, as well as, inflammatory response is regulated by PPAR, whose activation is triggered by poly-unsaturated fatty acids (PUFA) and fibrates [52]. Three mammalian PPARs identified are, PPAR-α, -δ and -γ. The agonists of these PPARs, mostly exert their anti-atherosclerotic properties by multiple mechanisms. They improve the systemic lipid levels, as well as, insulin resistance. PPARs also cause inhibition of the accumulation

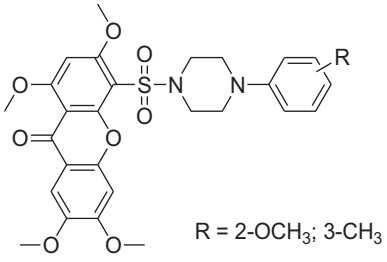

Fig. 18. Xanthone sulphonamides series.

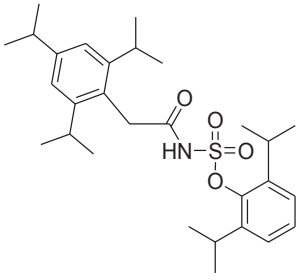

Fig. 20. Avasimibe.

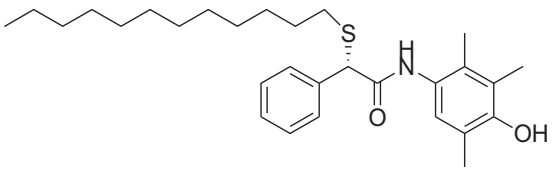

Fig. 21. Eflucimibe.

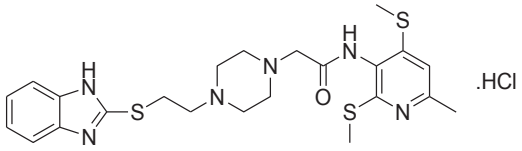

Fig. 22. K604.

of macrophage foam cells located in the endothelium, which is a crucial step in the formation of atherosclerosis.

PPAR-α is a potential target as its downregulation may alter vascular function and monocyte adhesion to aortic smooth muscle cells [53]. Furthermore, its activation enhances free fatty acid oxidation, controls expression of multiple genes regulating lipoprotein concentration and anti-inflammatory effects. Most importantly, it increases HDLC synthesis, stimulates RCT and reduces triglyceride absorption. Ursolic acid (Fig. 6), an indirect ligand of PPAR-α, regulates expression of lipid metabolism genes [54]. PPAR-α selective agonists are effective anti-atherosclerotic agents compared to PPAR-γ and liver X receptor (LXR) agonists [55].

PPAR-γ is also a potential target as, its activation causes the efflux of cholesterol from macrophage foam cells [56]. It is an active regulator of adipocyte differentiation, predominantly expressed in adipose tissue and is an important component in the adipogenic signalling cascade involved in lipid storage and utilization. Being atheroprotective, the activation of PPAR-γ can be promising in the treatment of atherosclerosis [57,58].

Activation of PPAR-δ can also be useful in the treatment of dyslipidemias and type-2 diabetes, as it improves insulin sensitivity and elevates HDL levels [59]. PPAR-δ appears to be implicated in regulating the burning of fatty acids at skeletal muscles and adipose tissue by controlling the expression of genes involved in fatty acid uptake, β-oxidation and energy uncoupling. Its ligand activation is associated with improved insulin sensitivity and elevated HDL cholesterol levels. Thus, PPAR-δ is a potential target for the treatment of obesity, dyslipidemias and type-II diabetes.

In conclusion, PPAR-δ, α and γ are good molecular targets for atherosclerotic therapy. Of these PPAR-δ is a better target for the treatment of obesity and dyslipidemia, while PPAR-α and PPAR-γ play complementary roles in the prevention of atherosclerosis.

Pleiotropic effects of the PPAR family are depicted in Fig. 7 [60].

Some important ligands of PPARs (Figs. 8–10) are listed in Table 3.

The phospholipid analogues of tetradecylthioacetic acid (TTA) (Fig. 11) have been found to be PPAR-α selective and effective in

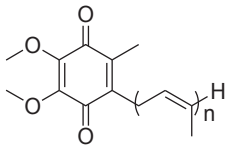

Fig. 24. Coenzyme Q10.

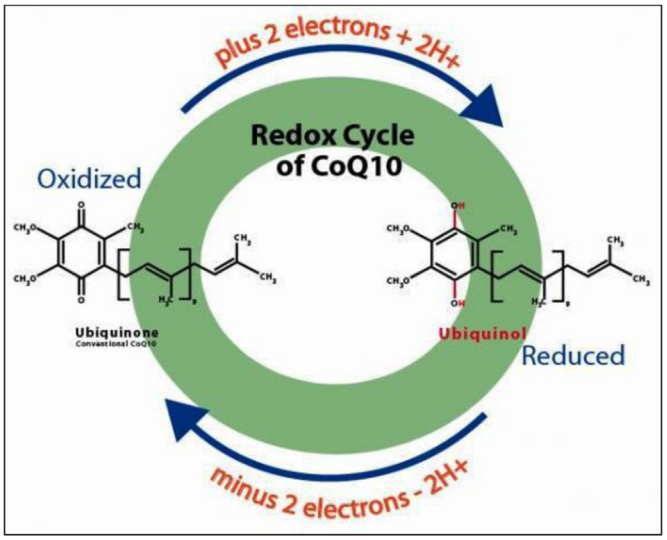

Fig. 25. Oxidation-reduction cycle of CoQ_{10}.

antidiabetic and antihyperlipidemic therapy [61]. By directly binding to all three PPAR subtypes cyanidin (Fig. 12) significantly reduced cellular lipid concentrations in lipid-loaded steatotic hepatocytes similar to hypolipidemic drugs [62].

The synthesis and structure–activity relationships of a novel series of α-aryloxyphenylacetic acids (Fig. 13) as PPAR-α/γ dual agonists were reported by G.Q. Shi et al. [63]. The co-crystal structures of one of the compounds from the series, as well as, the drug, rosiglitazone (Fig. 14) with PPAR-γ ligand binding domain (Fig. 15) were compared. The compounds were evaluated for their *in vivo* efficacy. They showed excellent anti-hyperglycaemic efficacy in a db/db mouse model and hypolipidemic activity in hamster and dog models, without provoking the typical PPAR-γ associated side effects in the rat tolerability assay.

4.2.1. In vitro biological evaluation protocol for PPAR-δ agonist activity

This assay is based on plasmid transfections for studying PPAR-δ activators in human carcinoma cells [64].

4.2.1.1. Cell culture and chemicals. A549 human lung adrenocarcinoma cell lines and HCT-116 human colorectal carcinoma cell lines are cultured in the media, RPMI-1640 and modified McCoy 5A,

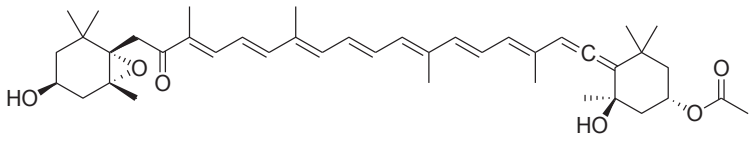

Fig. 23. Fucoxanthin.

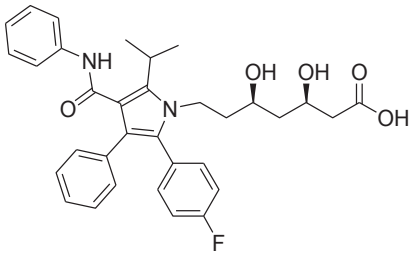

Fig. 26. Atorvastatin.

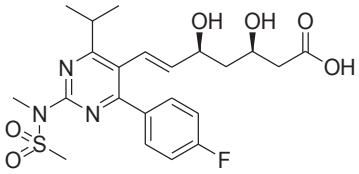

Fig. 27. Rosuvastatin.

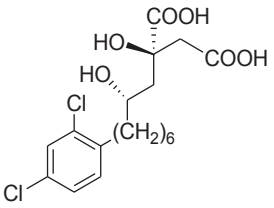

Fig. 29. SB-201076.

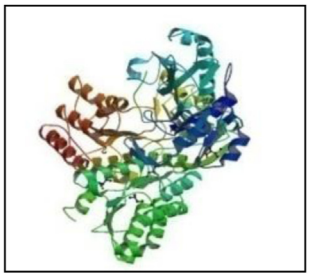

Fig. 30. Truncated human ATP citrate lyase with citrate bond – 3MWD.

respectively. Both the media are supplemented with 10% FBS and 101 g/ml gentamycin.

4.2.1.2. Transient transfections and luciferase reporter assays. Cells (1×10^5 cells/well) are cultured in twelve well plates in culture medium containing 10% FBS. After 16 h growth, the internal control, 0.05 μg pRL-null (renilla luciferase) (Promega, WI) and 0.5 μg of the other plasmids are transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's protocol. After 24 h, the media is changed and the cells are washed with PBS and harvested in 1X luciferase lysis buffer. The luciferase activity is measured by a dual luciferase assay kit (Promega, WI).

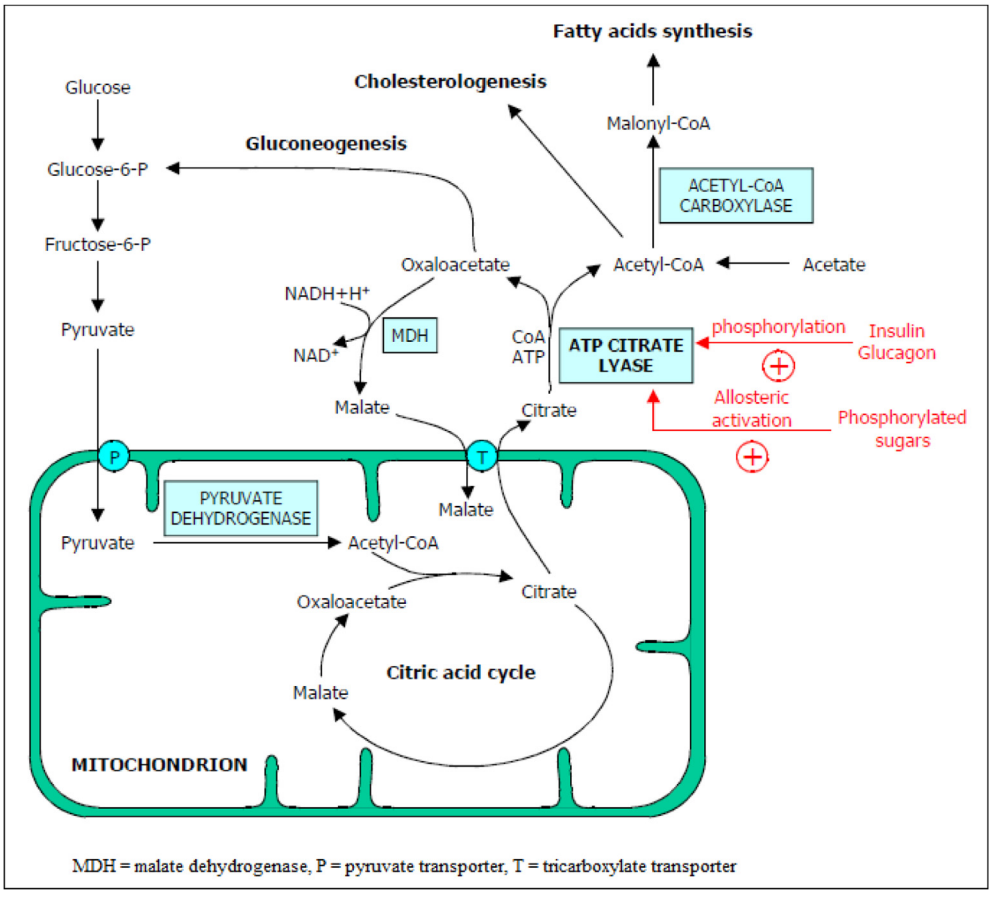

Fig. 28. Involvement of ACL in several biosynthetic pathways.

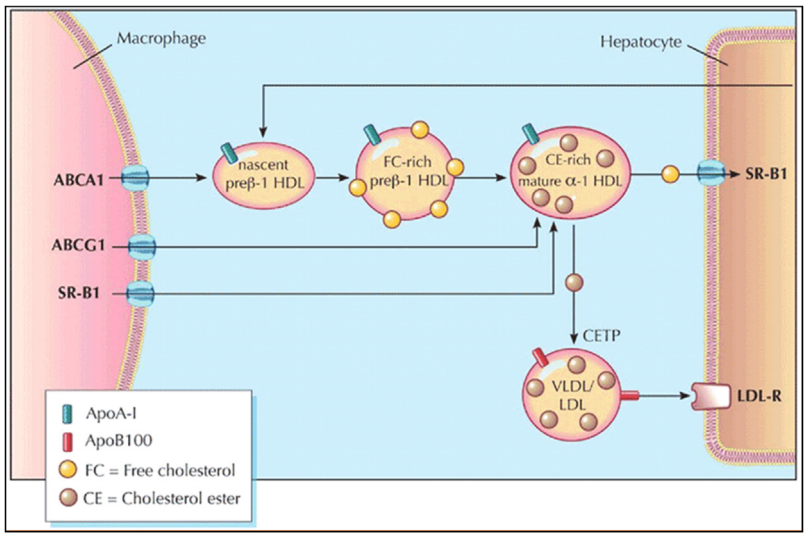

Fig. 31. Reverse cholesterol transport: HDLC promotes and facilitates the process of reverse cholesterol transport (RCT), in which extra macrophage cholesterol is effluxed to HDL and finally returned to the liver for excretion. Efflux to nascent and mature HDL occurs *via* the transporters ABCA1 and ABCG1, respectively. The HDL cholesterol is returned to the liver *via* the hepatic receptor SR-BI (Scavanger receptor class B-type-I) or by transfer to apolipoprotein (apo) B-containing lipoproteins by the action of CETP.

4.3. Acyl-CoA cholesterol acyl transferase (ACAT) [inhibitors of ACAT]

ACAT is an endoplasmic reticulum (ER) bound intracellular enzyme, which catalyzes the formation of cholesteryl esters (CE) from cholesterol and long chain fatty acyl CoA in a wide variety of cells [65]. During formation of macrophage derived foam cells, there is high level expression of ACAT-1, an isoenzyme essential for intracellular storage of cholesteryl esters. ACAT-2, another isoenzyme, expressed exclusively in small intestine and liver is also crucial for chylomicron assembly and cholesterol absorption (Fig. 16) [66].

ACAT inhibitors act by suppression of cholesterol absorption and suppression of foam cells formation in the arterial walls.

CE are storage forms of excess cellular cholesterol. In most cell types, CE are present only in low levels, mainly as cytoplasmic lipid droplets. In plasma, CE are part of the neutral lipid cargo present in the intestinal chylomicrons and in the hepatic VLDL [67]. In the atherosclerotic disorder, chronic accumulation of CE in macrophages causes these cells to appear foamy. It is a hallmark of early stages of atherosclerosis. For reasons described above, ACAT has been considered as a drug target for therapeutic intervention against atherosclerosis related disorders and can be blocked with suitable ligands [65–67].

In a study by T. Ohshiro et al. [68], atherogenic mice treated with pyriproprene A (PPPA), an ACAT inhibitor (Fig. 17) showed a decrease in intestinal cholesterol absorption and cholesteryl oleate levels of both LDL and VLDL, resulting in the protection from atherosclerosis development.

A series of xanthone sulphonamides (Fig. 18) as potent ACAT inhibitors, has been reported. Some of these compounds have proved to be more potent than the positive control, Sandoz-58-035 (Fig. 19) [69].

Anti-atherosclerotic activity of avasimibe (Fig. 20) and efluci-mibe (Fig. 21) is through the inhibition of ACAT in the arterial lumen [70]. Unlike the statins, strategic inhibition of ACAT can be applied clinically for reducing plasma cholesterol, which in turn reduces size of the lipid-rich core in the atherosclerotic plaques [71].

K604 (Fig. 22), a potent and selective ACAT-1 inhibitor developed by Yoshinaka et al. [72], significantly reduced macrophage positive areas in atherosclerotic plaques in the aorta indicating direct plaque modulating effects of K604 in apoE-knockout mice.

A study [73] on high fat diet fed C57BL/6N mice revealed that fucoxanthin (Fig. 23) significantly lowered hepatic lipid contents in the animals, by reducing the activity of hepatic lipogenic enzymes. Fucoxanthin also lowered blood glucose and HbA1c levels in the animals, along with plasma restin and insulin concentrations.

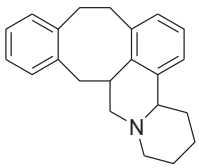

Fig. 32. Taclamin.

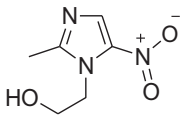

Fig. 33. Metronidazole.

4.3.1. In vitro biological evaluation protocol for ACAT inhibitory activity

The underlying principle involves the measurement of specific radioactivity of the microsomal proteins [74].

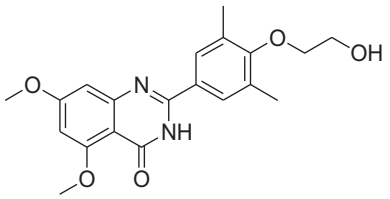

Fig. 34. RVX-208.

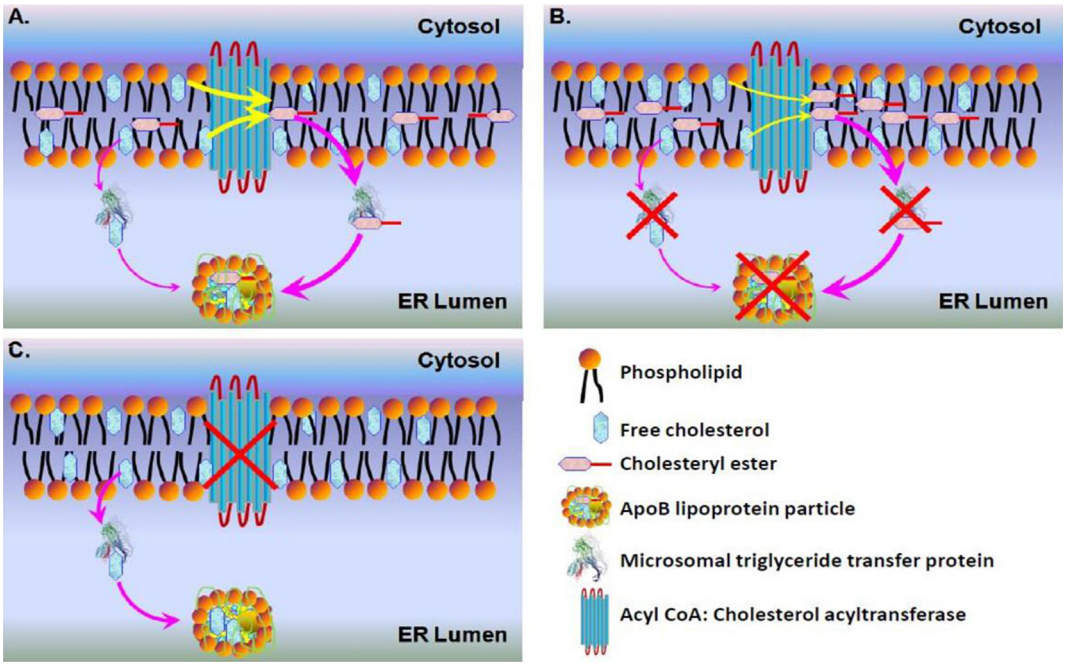

Fig. 35. Role of MTP in cellular cholesterol ester biosynthesis. (A) ACAT (yellow arrows) converts free cholesterol in the endoplasmic reticulum (ER) leaflets into cholesterol esters that remain within the membrane bilayer. MTP transfers both free cholesterol and cholesterol esters from the ER membranes to apoB-lipoproteins in the ER lumen. (B) In MTP deficient conditions, transfer of free and esterified cholesterol to apoB-lipoproteins is reduced. (C) MTP transfers free cholesterol to lipoproteins avoiding excess free cholesterol accumulation in the ER membrane. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

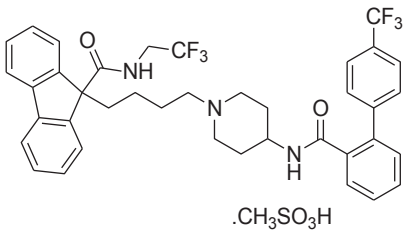

Fig. 36. Lomitapide mesylate.

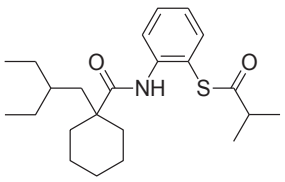

Fig. 37. Dalcetrapib.

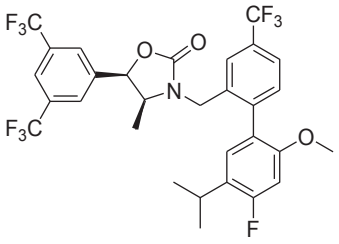

Fig. 38. Anacetrapib.

4.3.1.1. Isolation of tissue microsomes. African green monkey liver tissue is homogenized in isolation buffer with a Dounce homogenizer. The supernatant is removed and discarded. The pellet is again homogenized and resuspended in 1 ml of ACAT assay buffer (0.1 M K_2HPO_4, pH 7.4). The concentration of microsomal protein is measured by the method of O.J. Lowry et al. [75].

4.3.1.2. ACAT assay. Microsomes are thawed, an aliquot containing 200 μg of protein was mixed with 1 mg of 1% bovine serum albumin and 20 μL of a cholesterol-saturated solution of β-cyclodextrin. The final volume is brought to 300 μL. The sample is equilibrated in a 37 °C water bath for 30 min and then [^{14}C]oleyl-CoA is added to the

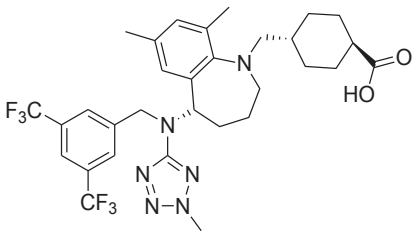

Fig. 39. Evacetrapib.

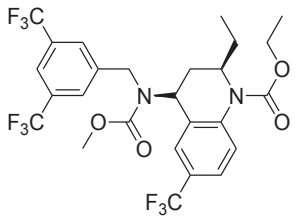

Fig. 40. Torcetrapib.

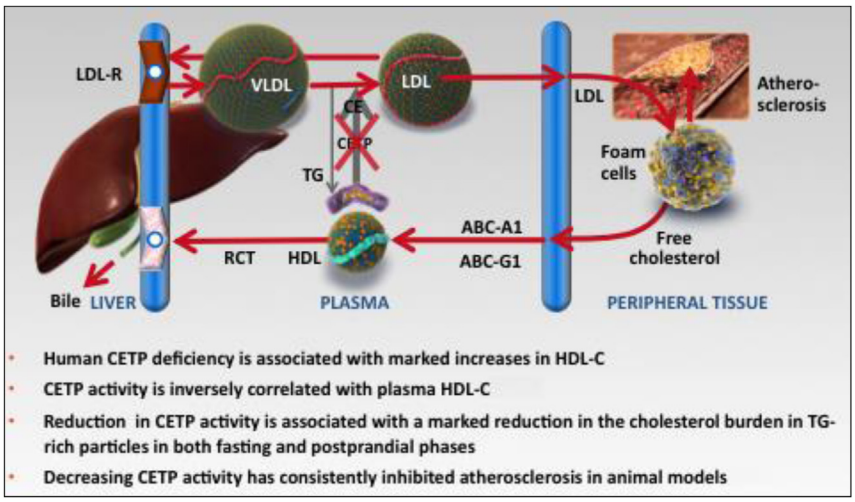

Fig. 41. Role of cholesteryl ester transfer protein (CETP) inhibition in atherosclerosis.

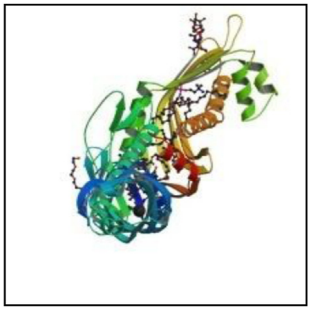

Fig. 42. Crystal structure of CETP in complex with inhibitors – 4EWS.

tube and incubated for 15 min. To stop the reaction, 6 ml of $CHCl_3$ – methanol [2:1] is added, followed by aqueous solution of KCl (1.2 ml). The sample is allowed to sit overnight at room temperature. The lower layer containing product is removed and evaporated to dryness under nitrogen. The residue is resuspended in 100 μl of $CHCl_3$ containing carrier lipid and then applied to a thin-layer chromatography (TLC) plate with subsequent separation in hexane-ethyl ether-acetic acid 70:30:1. The portion of the TLC plate containing the cholesteryl esters is scraped and suspended in scintillation fluid and radioactivity is determined.

4.4. Coenzyme Q10 (CoQ10) [stimulators of CoQ10]

Human heart depends on Coenzyme Q10 (Fig. 24) for its bio-energetic actions. CoQ10 is having a side chain with ten isoprenoid units and possesses antioxidant properties. Though it is found in all cell membranes (so also called as, ubiquinone), it is predominantly found in the myocardium [76].

Presence of CoQ10 in blood diminishes the oxidative stress of LDLC settings hence, CoQ10 is clinically effective in the prophylaxis and treatment of CVD's [77], atherosclerosis [78], angina [79] and congestive heart failure (CHF) [80].

The antioxidant nature of CoQ10 is derived from its energy carrier function. As an energy carrier, the CoQ10 molecule is

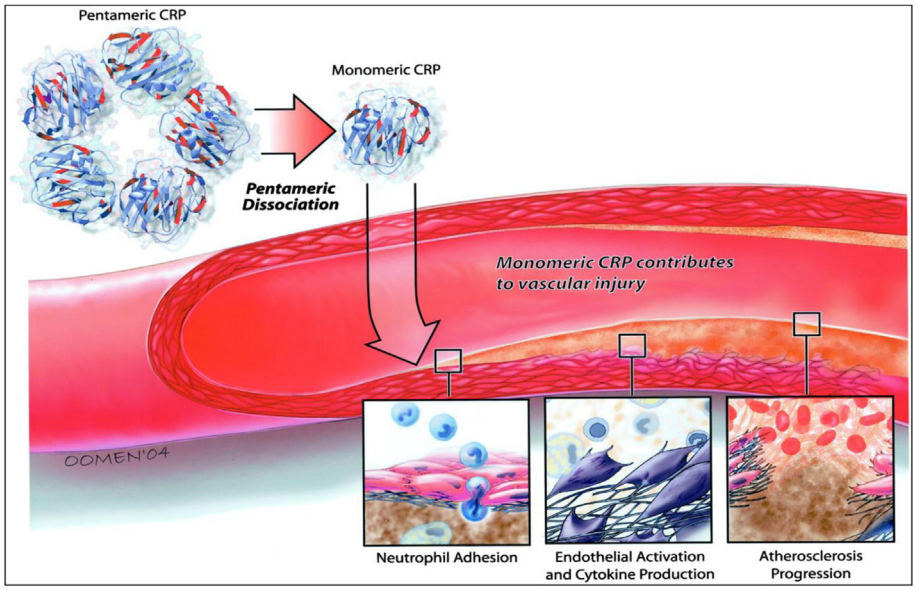

Fig. 43. Conversion of pentameric form of C-reactive protein into monomeric form.

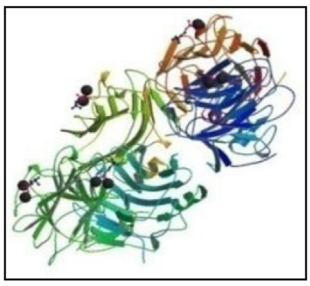

Fig. 44. Structure of human CRP complexed with phosphocholine-1B09.

continually going through an oxidation–reduction cycle (Fig. 25). As it accepts electrons, it becomes reduced and as it gives up electrons, it becomes oxidized. In its reduced form, the CoQ10 molecule holds electrons rather loosely, so it can quite easily give up one or both electrons and thus, act as an antioxidant. CoQ10 inhibits lipid peroxidation by preventing the production of lipid peroxyl radicals. Moreover, its reduced form reduces the initial perferryl radical and singlet oxygen, with concomitant formation of ubisemiquinone and H_2O_2. Thus, by virtue of its antioxidant effect, CoQ10 can negatively control lipid peroxidation and thereby hamper the atherosclerotic plaque formation.

This quenching of the initiating perferryl radicals prevents the propagation of lipid peroxidation and protects not only lipids, but also proteins from oxidation. In addition, the reduced form of

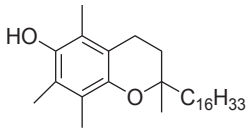

Fig. 46. Vitamin E.

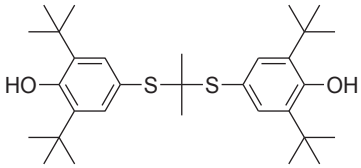

Fig. 47. Probucol.

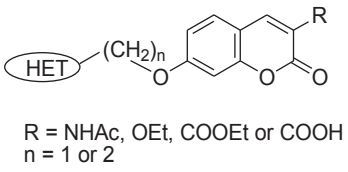

Fig. 48. Coumarin derivatives.

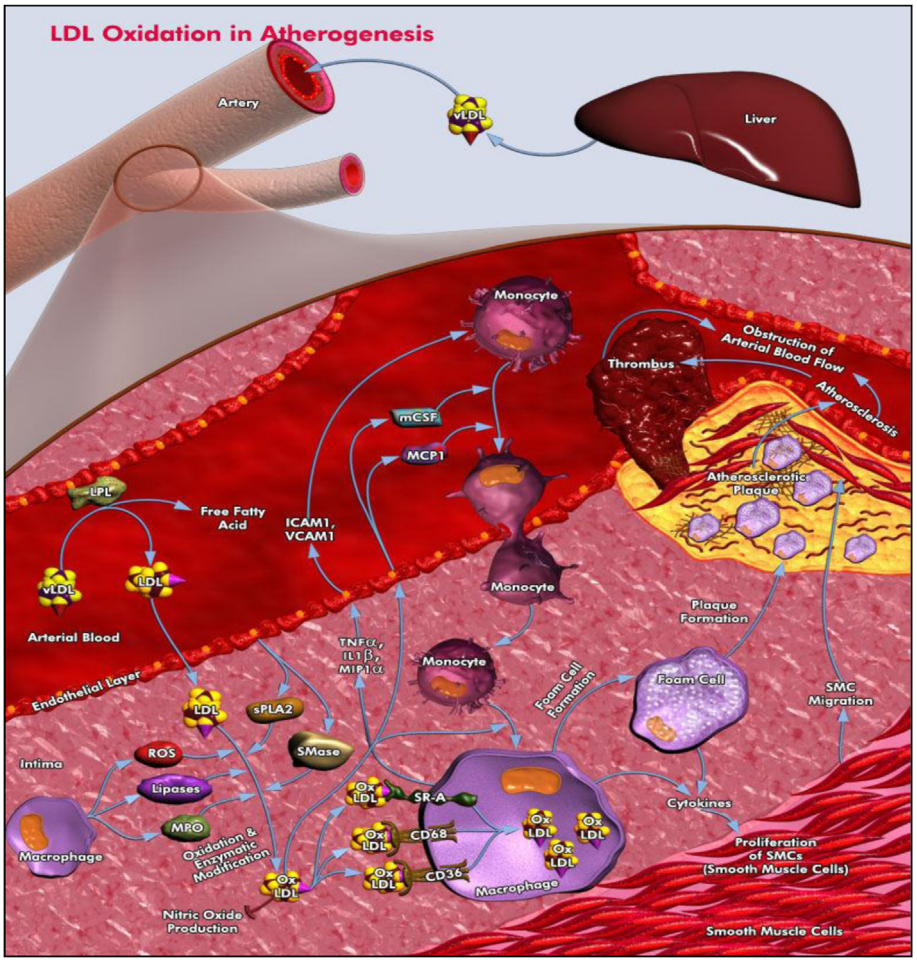

Fig. 45. Lipid oxidation in atherogenesis.

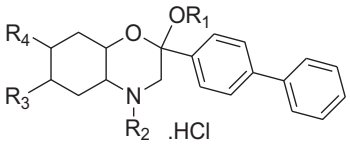

Fig. 49. 2-Biphenylmorpholine derivatives.

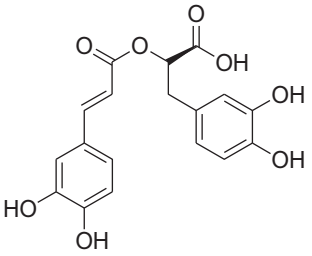

Fig. 50. Rosmarinic acid.

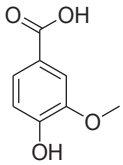

Fig. 51. Vanillic acid.

CoQ10 effectively regenerates vitamin E from the α-tocopheroxyl radical, thereby interfering with the propagation step. Furthermore, during oxidative stress the interaction of H_2O_2 with metal ions bound to DNA generates hydroxyl radicals leading to the oxidation of bases, in particular, in mitochondrial DNA, CoQ10 efficiently prevents this oxidation. In contrast to other antioxidants, this compound inhibits both the initiation and the propagation of lipid and protein oxidation. It also regenerates other antioxidants such as vitamin E. The circulating CoQ10 in LDL prevents oxidation of LDL, which may be beneficial in CVDs [81].

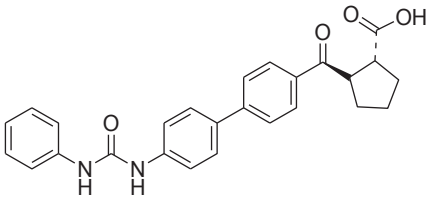

Fig. 53. A-922500.

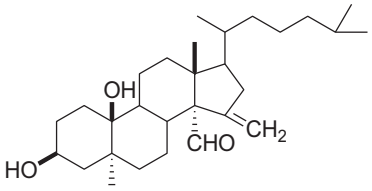

Fig. 54. Ethenyllanosterol.

C. Schmelzer et al. [82], have analysed the effects of reduced form of CoQ10 on cholesterol metabolism at the transcriptional and metabolite levels in SAMP1 mice.

B.I. Ognjanović et al. [83], have successfully investigated the protective role of CoQ10 (20 mg/kg) and vitamin E (20 IU/kg) alone or in combination against cadmium (Cd, 0.4 mg/kg) induced lipid peroxidation and changes in antioxidant defence system in the rat testes.

Statins are among the most widely used drugs in the management of hypercholesterolaemia. Though, they inhibit the endogenous cholesterol synthesis, they also decrease CoQ10 synthesis. As CoQ10 has antioxidant properties, administration of such drugs that decrease CoQ10 synthesis, might lead to the increased oxidative stress *in vivo*. Liu et al. [84], have demonstrated atorvastatin (Fig. 26) to exert its hypocholesteromic effects without interfering with CoQ10 levels and oxidative stress in hypercholestermic patients. The atorvastatin therapy attenuated myocardial necrosis

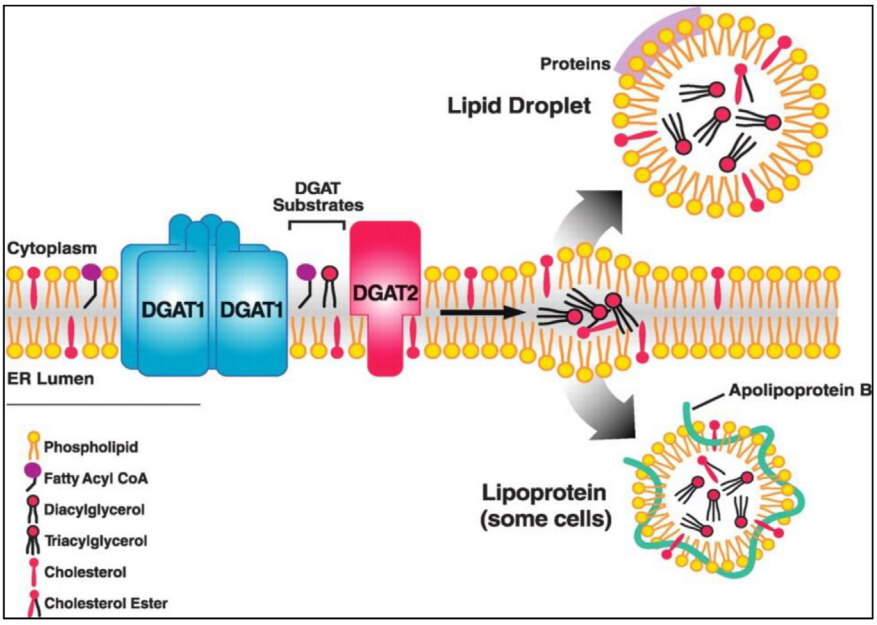

Fig. 52. Role of DGAT in triglyceride production.

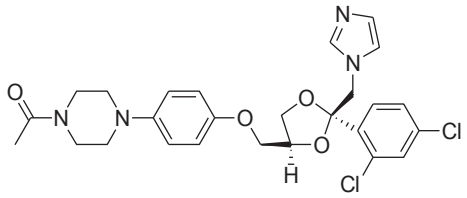

Fig. 55. Ketoconazole.

and fibrosis in isoproterenol-induced heart failure. However, a high dose of the drug considerably worsened the left ventricular dysfunction and haemodynamic depression, which was reversed by CoQ10 co-administration [85].

K. Toyama et al. [86], have compared the effects of two statins, atorvastatin and rosuvastatin (Figs. 26 and 27) combined with exercise, on CoQ10 and HDLC levels in twenty eight patients with coronary artery disease (CAD). Though, both statins equally improved exercise capacity and lowered the LDLC and triglyceride levels, rosuvastatin preserved the CoQ10 levels. Thus, rosuvastatin with regular exercise could be beneficial for CAD patients.

At a dose of 150 mg CoQ10 supplements can decrease oxidative stress and increase antioxidant enzyme activity in patients with CAD. A higher dose of CoQ10 supplements (>150 mg/day) might promote rapid and sustainable antioxidation in the patients [87].

Thus, ligands stimulating the production and action of CoQ10 can be useful in antihyperlipidemic therapy.

4.4.1. *In vitro biological evaluation protocol for potentiation of antioxidant effects of CoQ10*

The underlying principle for this assay is of the alkaline microgel electrophoresis (Comet assay) and reduction of DNA damage (as antioxidant effect) which is compared to H_2O_2 induced damage [88].

4.4.1.1. Incubation. Mini organs are incubated with two concentrations of CoQ10 (1 μM and 10 μM, respectively) dissolved in distilled water for 60 min. After incubation, the cultures are exposed to oxidative stress using H_2O_2 at a concentration of 1 mM for an exposure time of 1 h. Thereafter, the cultures are suspended in bronchial epithelial cell growth medium (BEGM; Promocell, Heidelberg, Germany).

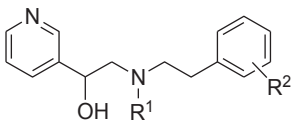

Fig. 56. Pyridylethanol(phenylethyl)amines.

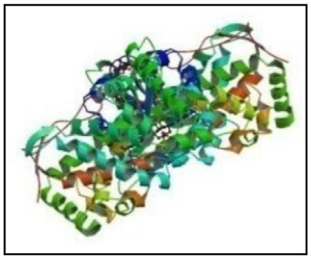

Fig. 57. Crystal structure of human lanosterol 14α-demethylase (CYP51) – 3JUV.

4.4.1.2. Comet assay. The samples undergo enzymatic digestion with collagenase P (1 mg/ml), hyaluronidase from bovine testes (1 mg/ml; Boehringer, Mannheim, Germany) and protease E type XIV from *Streptomyces griseus* (5 mg/ml; Sigma, Steinheim, Germany) for 45 min in a 37 °C shaking water bath. The alkaline microgel electrophoresis assay (Comet assay) is used to measure DNA single strand breaks and alkaline labile sites. Antioxidant effects of CoQ10 are evaluated using concentrations of 1 μM and 10 μM.

4.5. *ATP citrate lyase (ACL) [inhibition/antagonist of ACL]*

ATP citrate lyase (ACL) is the key lipogenic enzyme that converts citrate in the cytoplasm to acetyl-CoA, which is a vital building block for the biosynthesis of fatty acids, cholesterol and isoprenoid [89–91].

Inhibition of ACL, which is involved in several biosynthetic pathways (Fig. 28), reduces plasma LDLC by inhibiting cholesterol synthesis and decreases plasma triglyceride levels by reducing fatty acid synthesis. Levels of hepatic free fatty acids available for triglyceride and VLDL synthesis are also reduced by inhibition of ACL. Thus, ACL is a good molecular target for controlling the plasma lipid levels, especially those of LDL, VLDL and triglyceride.

A series of ACL inhibitors have been identified by N.J. Pearce et al. [92]. One of the compounds, SB-201076 (Fig. 29) has exhibited potent ACL inhibitory activity and is being studied as novel antihyperlipidemic agent.

To explore the physiological actions of hepatic ACL in lipid homoeostasis in response to nutritional changes in mice fed with a low-fat diet *versus* those with a high-fat diet, Q. Wang et al. [93], in an experiment specifically knocked down the hepatic ACL expression by adenovirus-mediated RNA interference in mice. Their results demonstrated that hepatic ACL suppression exerts profound effects on triglyceride mobilization, as well as, fatty acid compositions in the liver, suggesting an important role for ACL in lipid metabolism.

R. Lin et al. [94], have discussed the difference between acetylation and ubiquitylation by competing for the same lysine residues in the regulation of fatty acid synthesis and cell growth in response to glucose. Increased fatty acid synthesis is required to meet the demand for membrane expansion of rapidly growing cells. Inhibition of ACL is not only a promising antihyperlipidemic approach but, it also has a hidden potential for the treatment of susceptible cancer cells.

ACL as a cytoplasmic enzyme plays an important role of linking energy metabolism from carbohydrates to the production of fatty

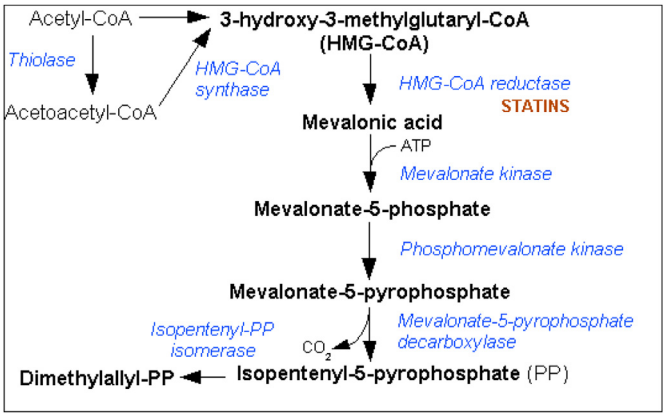

Fig. 58. Role of HMG-CoA synthase.

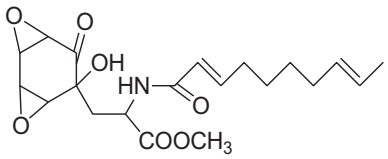

Fig. 59. *Trans*-DU-6622.

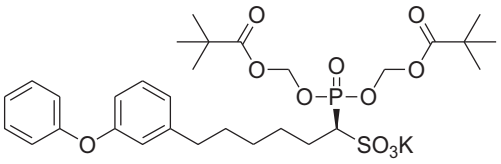

Fig. 63. BMS-188,494.

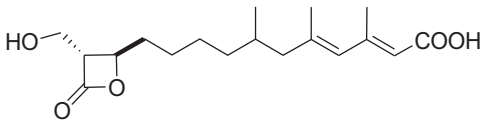

Fig. 60. L-659,699.

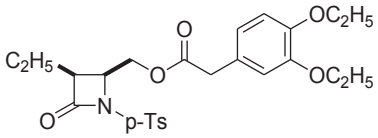

Fig. 61. Tosyl lactams.

acids. *In situ* proteolysis of full-length human ACL yielded crystals of various conformations of the fragment residues of an 1101-amino acid protein. These crystals were grown in the presence of tartrate or the substrate, citrate, and the structure revealed the citrate-binding site of human ACL as deduced by X-ray crystallography (Fig. 30) [95].

4.5.1. *In vitro biological evaluation protocol for ACL inhibitory activity*

This assay is based on the estimation of radioactivity of human ACL and quantifying [^{14}C] acetyl CoA signal [96]. The protocol for this assay is as follows:

The enzymatic reaction of purified human ACL is carried out in 20 μL of buffer D (87 mM Tris, pH 8.0, 20 μM MgCl, 10 mM KCl, 10 mM DTT) containing substrates 100 μM CoA, 400 μM ATP, 150 μM [^{14}C] citrate (specific activity: 2 μCi/μmol) in a 384-well polyplate at 37 °C for 3 h. The reaction is terminated by the addition of 1 μl 0.5 M EDTA solution to reach ~24 mM final concentration. An aliquot of 60 μl MicroScint-O is then added to the reaction mixture and incubated at room temperature overnight with gentle shaking. The [^{14}C] acetyl CoA signal is detected in a TopCount NXT liquid scintillation counter. The count time is 1 min/well and the unit of signal is expressed as counts per minute (CPM). [μCi is a unit or quantity of radiation equivalent to one-millionth of a curie].

4.6. Concentration of HDL levels [enhancers of HDL]

High concentration of HDL in the body results in antiatherogenic effects due to clearance of free cholesterol from cells and delivering it to the liver. Such activity has been correlated to the decrease in the number of sites of coronary arteries stenosis [97]. HDL prevents the progression of atherosclerosis, through several mechanisms. The antiaetherothrombotic effects of HDL are well established (Figure S1, supplementary material) [98].

Promotion of cholesterol efflux from macrophages and its return to the liver, bile, feces and completing the pathway of reverse cholesterol transport (RCT), is thought to be one of the most important mechanisms by which HDL prevents the deposition of cholesterol and thereby the progression of atherosclerosis (Fig. 31) [99].

The ability of RCT by clearing cholesterol from the arterial wall is antiatherogenic in nature [98,100]. After a fat load, in

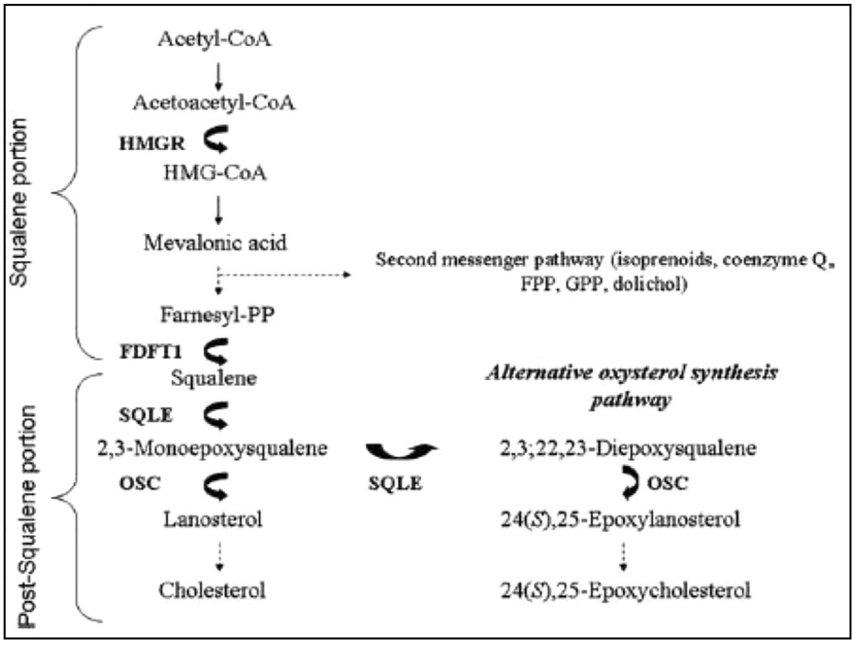

Fig. 62. Cholesterol synthetic pathway divided into – squalene and the post-squalene portions.

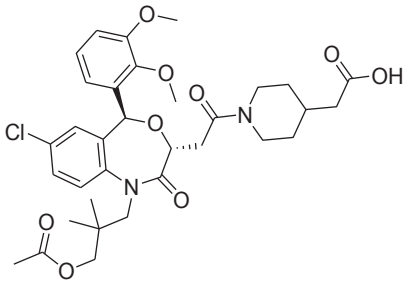

Fig. 64. Lapaquistat acetate.

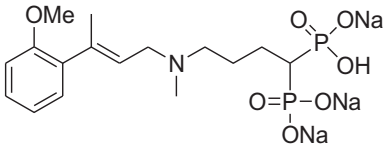

Fig. 65. ER-28448.

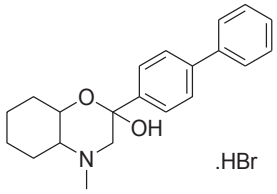

Fig. 66. EP 2306.

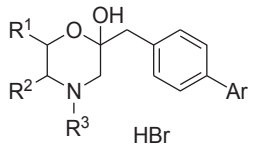

Fig. 67. Morpholine derivatives.

normolipidemic subjects, HDL concentration usually remains unchanged [101–104] or decreases slightly [105].

Taclamin (Fig. 32) is one of the non-sulphur compounds which enhance HDL levels. Similarly, the biogenic LCAT, hepatic lipase and phospholipid transfer proteins (PLTP), all increase the plasma HDL subpopulations [106].

By increasing HDLC levels, metronidazole (Fig. 33) acts as anti-atherogenic in experimental animals. D. Bailey et al. [107], have reported a quinazoline; RVX-208 (Fig. 34) to be useful for the treating atherosclerosis, as it induced apoA-I mRNA and protein synthesis in HepG2 cells, leading to increased levels of pre-β-migrating and α-lipoprotein particles containing apoA-I (LpA-I) in spent media.

Being natural nanoparticles, lipoproteins can be useful in delivering imaging agents. Importantly, their endogenous nature makes them biocompatible and biodegradable and allows them to avoid the recognition by the reticuloendothelial system. In particular, HDL particles are of interest. Because of their small size they can easily cross the endothelium and penetrate the underlying tissue. By loading various types of image-enhancing compounds into either the core or surface of HDL, they can be visualized by different imaging modalities (MRI, CT, optical, etc.). By rerouting of HDL away from plaque macrophages, imaging of biological processes in diseases besides atherosclerosis may also be achieved [108].

Many clinical and preclinical studies have established relations between elevated HDL levels and their anti-atherogenic properties [109] and have also highlighted the inverse relationship of HDLC with cardiovascular mortality [110].

Atherosclerosis increases markedly in hypercholesterolemic mice deficient in apolipoprotein (apoA)-I, the major HDL protein, whereas overexpression of human apoA-I dramatically retards atherosclerosis in hypercholesterolemic mice. Taken together, these findings provide strong evidence that apoA-I plays a key role in HDL's cardioprotective activities. These observations have triggered off intense interest in targeting HDLC for therapeutic intervention due to lowering LDL levels [111].

4.6.1. In vitro biological evaluation protocol for HDL enhancer activity

This assay is based on the ability of HDL to reverse oxidation of LDL using 2,7-dichlorofluoresceindiacetate (DCFH-DA) and a copper-oxidized LDL preparation. DCFH-DA is dissolved in fresh methanol at 2.0 mg/ml and incubated at room temperature in dark for 30 min to release DCFH. Its interaction with lipid oxidation products produces intense fluorescence. Fluorescence intensity is measured using a fluorescent plate reader set at an excitation wavelength of 485 nm and an emission wavelength of 530 nm [112].

4.7. Microsomal triglyceride transfer protein (MTP) [inhibitors of MTP]

Transport of lipid molecules between phospholipid membranes is catalysed by MTP. MTP also transfers triglycerides during VLDL assembly. Hence, inhibiting MTP leads to reduction in packaging

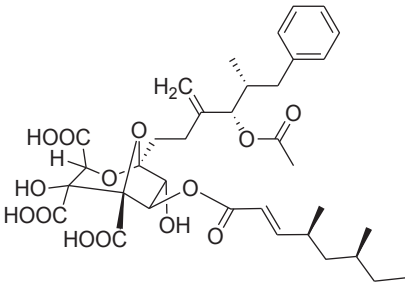

Fig. 68. Zaragozic acids-A.

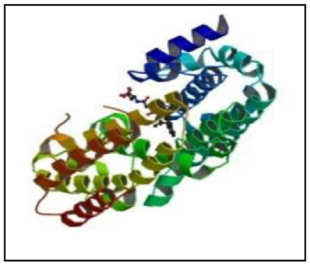

Fig. 69. Crystal structure of human squalene synthase – 1EZF.

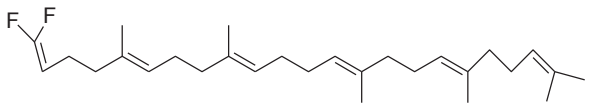

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Fig. 70. 1,1-Difluorosqualene.

and secretion of VLDL and chylomicrons. This actually contributes to the antiatherogenic activity of MTP inhibitors [113].

MTP is required for the secretion of apo B-containing lipoproteins from hepatocytes, as well as, the absorptive enterocytes of the intestine [114,115]. MTP is also found in other tissues such as the myocardium [116,117], yolk sac [118] and kidneys [119].

The assembly of lipoproteins in the liver begins with the synthesis of apolipoprotein B (apoB) molecules. Newly synthesized apoB within the ER lumen may undergo one of the two fates based on lipid availability. In the presence of sufficient lipid and MTP activity, apoB is assembled into a lipoprotein. Hepatic VLDL production is in turn a key underlying factor for the development of the metabolic dyslipidemia accompanying insulin resistance [120].

M.M. Hussain et al. [121], have reviewed the roles that MTP plays in the assembly of apoB-lipoproteins, the regulation of CE synthesis, biosynthesis of CD1 proteins and propagation of hepatitis C virus (Fig. 35).

The role of leptin signalling in MTP regulation and lipid absorption using several mouse models deficient in leptin receptor (LEPR) signalling and downstream effectors has been studied. Mechanistic studies revealed that the accumulation of triglyceride in the intestine might be secondary to decreased expression of MTP and lipid absorption in these mice [122].

Study on high fat diet fed hamsters and mice [123], has demonstrated, hepatic triglyceride secretion and MTP expression to be associated with plasma FFA levels and cholesterol biosynthesis, but not hepatic steatosis.

Recently, a novel antihypercholesteremic drug, lomitapide mesylate (Juxtapid™) (Fig. 36) introduced by Bristol-Myers-Squibb as a MTP inhibitor, has been approved by US FDA for the treatment of patients with familial hypercholesteremia [124].

4.7.1. In vitro biological evaluation protocol for MTP inhibitory activity

The underlying principle involves the study of triglyceride transfer activity in rat hepatocytes [113].

Pure bovine liver MTP (10 mg) is incubated with various concentrations of 2-[1-(4-benzoylphenyl)-4-piperidinyl]-2,3-dihydro-1*H*-isoindol-1-one (BMS-192951), test compound to be evaluated, for 1 h at room temperature in a final volume of 100 ml of assay buffer (15 mm Tris–HCl, pH 7.5, 40 mm NaCl, 1 mm sodium EDTA, and 0.02% sodium azide) in opticlear glass vials (Kimble). The samples are irradiated with ultraviolet (UV) light by placing them on the top of an ultraviolet transluminator box (360 nm, UVP, San Gabriel, CA, Model TL-33E, 115 V, 60 Hz, 1.8 Amps) for 5 min at 4 °C. The temperature of the sample is less than 30 °C after the UV exposure. The samples are then dialysed overnight in assay buffer at 4 °C to remove unbound inhibitor and triglyceride transfer activity is determined.

4.8. Cholesteryl ester transfer protein (CETP) [inhibition of CETP]

Cholesteryl ester transfer protein (CETP), also known as plasma lipid transfer protein is a plasma protein (mainly bound to HDL) that facilitates the transport of cholesteryl esters and triglycerides between the lipoproteins. Liver secrets CETP which during circulation, transfers cholesterol from HDL cholesterol to VLDL or LDL. This results in lowering the levels of HDL in blood, manifesting into risk of atherosclerosis. Redistribution of CE, as well as, TGs is promoted by CETP. Thus, CETP plays an important role in the management of atherosclerosis and hence a target where inhibition can lead to elevation of HDL levels, thereby prevention of atherosclerosis [125,126].

M.J. Chapman et al. [127], have reported evidence for a central role of CETP in the action of current lipid-modulating agents with HDL-raising potential, *i.e.*, statins, fibrates, niacin and compared their mechanisms of actions with those of pharmacological agents under development as CETP inhibitors. New CETP inhibitors, such

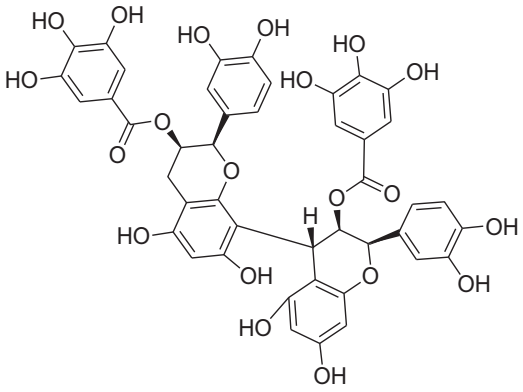


Fig. 71. Procyanidin B-2 3,3′-di-*O*-gallate.

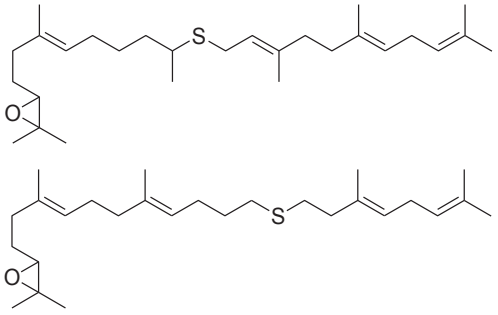


Fig. 72. Sulphur-substituted oxidosqualene analogues.

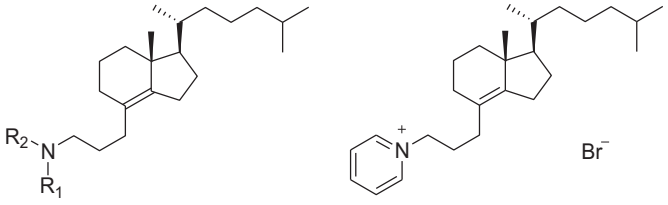


Fig. 73. Aminopropylindenes.

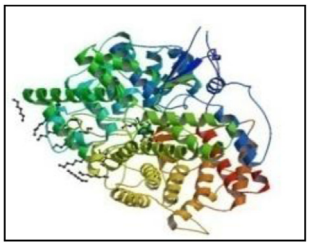


Fig. 74. Crystal structure of Squalene 2,3-oxide-lanosterol cyclise – 1W6K.

as dalcetrapib (Fig. 37) and anacetrapib (Fig. 38), are targeted to normalize HDL/apoA-I levels and stimulate the anti-atherogenic activities of HDL particles.

Thus, CETP is considered as a potential target to treat dyslipidemia and this hypothesis is supported by many animal studies [128]. S.J. Nicholls et al. [129], have clinically examined significant reductions in LDLC when a CETP inhibitor is administered in combination with statins.

B.J. Kappelle et al. [130], have discussed the balance between possible benefits and harms related to the pharmacological CETP inhibition.

G. Cao et al. [131], have described a novel benzazepine compound, evacetrapib (Fig. 39) (LY2484595) which is a potent and selective inhibitor of CETP both *in vitro* and *in vivo*. This compound, chemically, *trans*-4-({(5*S*)-5-[{[3,5-bis(trifluoromethyl)phenyl] methyl}(2-methyl-2*H*-tetrazol-5-yl)amino]-7,9-dimethyl-2,3,4,5-tetrahydro-1*H*-benzazepin-1-yl}-methyl)cyclohexanecarboxylic acid, is presently a drug under development. It is thought that modifying lipoprotein levels modifies the risk of CVD. In double transgenic mice expressing human CETP and apoA-I, evacetrapib exhibited an *ex vivo* CETP inhibition at an ED_{50} of less than 5 mg/kg at 8 h *p.o.*, dose and significantly elevated HDLC levels. Importantly, no blood pressure elevation was observed in rats dosed with evacetrapib at high exposure multiples as compared to the positive control, torcetrapib (CP-529,414, Pfizer) (Fig. 40). Another molecule developed till phase III clinical trials but, withdrawn in 2006.

J.G. Robinson [132] has presented an overview of phase II data of another CETP inhibitor, dalcetrapib (JTT-705) which has a unique chemical structure (IUPAC name, *S*-[2-({[1-(2-ethylbutyl)cyclohexyl]carbonyl}amino)phenyl]-2-methylpropanethioate) and it induces a conformational change in CETP, rather than forming a non-productive CETP/HDLC complex as do the other CETP inhibitors. Though, the molecule under development by Hoffmann–La Roche, did advance to phase III clinical trials, its development was halted on May 7, 2012 due to a "lack of clinically meaningful efficacy".

CETP reduces circulating HDL levels by transferring CE from HDL to larger lipoproteins, such as chylomicrons, VLDL and LDL, in exchange for triglyceride (Fig. 41).

Though, it creates a smaller, cholesterol-depleted HDL (remodelling), which is potentially beneficial in removing excess tissue cholesterol, it also creates a small, cholesterol-depleted LDL (SD-LDL), which is highly atherogenic.

Inhibiting the latter without impairing HDL remodelling may be critical to the success of CETP inhibitors [133]. A trifluoro-3-amino-2-propanol, is reported to inhibit the CETP-mediated transfer of [^{3}H]-CE from HDL to LDL in human plasma (IC_{50} = 0.6 μM) [134,135].

S. Lui et al. [136], have reported the crystal structure of CETP in complex with torcetrapib (Fig. 42), showing unusual inhibition mechanism (tunnel mechanism) for neutral lipid transfer by CETP. An enhanced understanding of the inhibitor binding site may provide opportunities to design novel CETP inhibitors possessing more drug-like physical properties, distinct modes of action, or alternative pharmacological profiles.

4.8.1. *In vitro biological evaluation protocol for CETP inhibitory activity*

This bioassay is based on the principle of enzyme linked immunosorbent assay using monoclonal human CETP antibody in whole plasma [137].

HDL labelled with [^{3}H]CE is prepared after whole plasma incubation with [^{3}H]cholesterol as described by Tall et al. [138] After overnight incubation, more than 97% of the [^{3}H]cholesterol radioactivity is isolated in the CE band produced by TLC. CETP activity is determined as the percentage of labelled CE transferred in 1 h at 37 °C from isolated human HDL to a mixture of human VLDL and LDL added in excess [139]. All assays are conducted in duplicate. CETP mass is measured by a two-site sandwich enzyme linked immunosorbent assay using monoclonal human CETP antibody (TP20) for coating the microtitre plate as the capture antibody. A second antibody (TP2), conjugated with horseradish peroxidase, is added to decorate the CETP antigen. Plasma dilutions in the range of 1:5 to 1:50 were made and 200 ml is then added to the plate for detection of CETP. Colour is detected using H_2O_2 as substrate and *o*-dianisidine as the chromogen [140].

4.9. *C-reactive protein (CRP) [inhibition of CRP]*

CRP is an acute phase reactant largely produced by the liver in response to inflammatory cytokines, such as interleukin-6, a marker of low grade vascular inflammation. CRP is a powerful independent predictor of future cardiovascular risks in any individual [141]. This discovery reflects the pivotal role that the pro-inflammatory processes play in atherogenesis and its complications [142]. Blood CRP circulates as a pentamer (Fig. 43). One face of this pentamer supports multipoint attachment to ligands and ligand-decorated surfaces; the other face binds C1q and FcγR. CRP is a member of the pentraxin family which consists of five non-covalently associated peptides surrounding a central core binding of bacterial and fungal polysaccharides. SAA (Serum amyloid A) is an apoprotein, associated primarily with HDL, inducing matrix degrading enzymes and acting as a chemo-attractant for monocytes, as well as, mediating lipid delivery to peripheral cells and removal of cholesterol from damaged tissues [143].

Cardiovascular risks like myocardial infarction, ischaemic stroke, sudden cardiac death, incident peripheral vascular disease and stenosis after percutaneous coronary intervention can be predicted by CRP levels as it is involved in the immunologic process that triggers vascular remodelling and plaque deposition [144].

HDL particle with serum amyloid A (SAA) attached to proteoglycan suggest the existence of inflammatory HDL [145]. P. Kostakou et al. [146], have determined and compared the pleiotropic effects of simvastatin and ezetimibe in dyslipidaemic patients. Results suggest that CRP lowering may occur in conjunction with LDLC lowering and not through a specific statin pleiotropic anti-inflammatory effect.

P.S. Sever et al. [147], have done a study to determine whether baseline CRP, on-statin CRP, as well as, on-statin-LDLC combination CRP are independent predictors of cardiovascular outcome. In their study on nearly 500 subjects, cardiovascular risk increased with baseline CRP (hazard ratio [HR] per subject; SD: 1.21; 95% confidence interval [CI]: 1.09–1.33) in an adjusted model. Though the baseline CRP independently predicted cardiovascular event risk, the achieved CRP levels in patients' continuing statin therapy either alone or in combination with LDLC, did not predict cardiovascular events, conclusively.

A multivariate analysis study has reported that higher CRP levels are associated with older age, female gender, hypertension, smoking, greater body mass index, larger waist circumference, LDLC levels, and triglyceride/HDLC ratio. In contrast, Asians taking a statin are associated with lower CRP levels [148].

The effects of three lipid-modifying therapies on LDLC, CRP levels, markers of cholesterol absorption and synthesis have been evaluated [149]. Statins alone or in combination with ezetimibe reduced the CRP levels. However, ezetimibe alone did not affect the CRP levels.

CRP concentration was found to decrease in a dose-dependent manner for lutein supplementation and serum CRP was directly related to the change in plasma lutein. Lutein supplementation

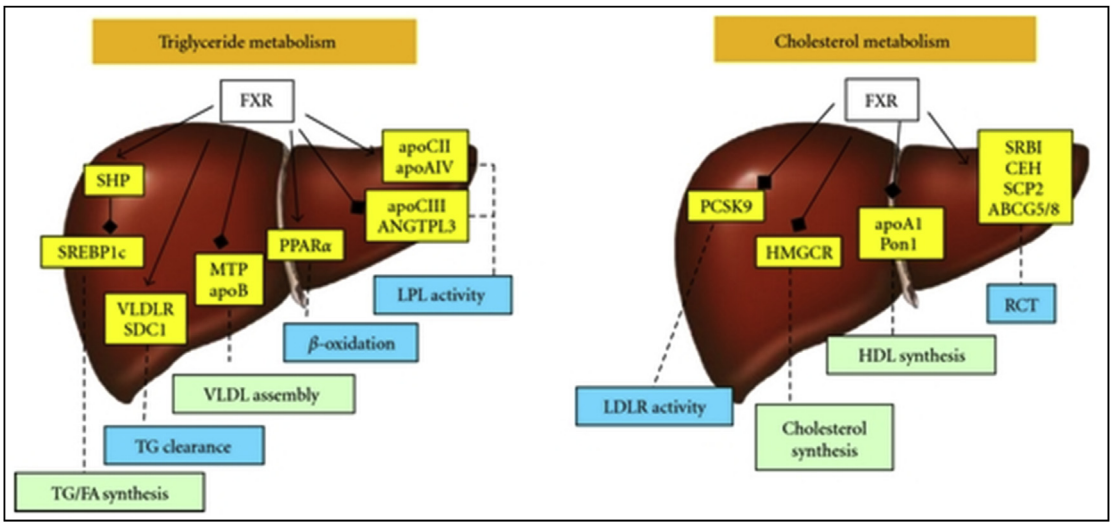


Fig. 75. FXR activation leads to reduced trans hepatic flux of bile acids thereby, decreasing LDL-C and increases HDL-C as well as, VLDL triglyceride levels.

reduces biomarkers of CVD risk *via* decreased lipid peroxidation and inflammatory response by increasing plasma lutein concentrations and antioxidant capacity [150].

Statins are known to reduce plasma CRP concentrations. Maximal doses of atorvastatin lowered plasma CRP levels substantially [151].

D. Thompson et al., have discussed the physiological structure of human CRP and its complex phosphocholine (PC) (Fig. 44). The structure shows how large ligands containing PC may be bound by CRP *via* phosphate oxygen that projects away from the surface of the protein. These findings may help in design of inhibitors of CRP binding that may have therapeutic relevance to the possible role of CRP in atherothrombotic events [152].

4.9.1. In vitro biological evaluation protocol for CRP

The underlying principle involves the CRP detection using a CRP antibody [153]. General procedure for assay is as follows:

Calibrators are constructed that contain isolated CRP at concentrations of 0.05, 0.10, 0.25, 0.50, 1.00, 5.00, and 10.00 mg/l in solution in 0.14 mol/l NaCl, 0.01 mol/l Tris, 0.002 mol/l $CaCl_2$ (pH 8.0), containing 10 g/l bovine serum albumin and 2 ml/l Tween 20 (TCBT buffer). After incubation at 37 °C for 1 h, the plates are decanted and each well is washed three times with 200 ml of TCBT buffer. Captured CRP is then detected by addition to each well of 100 ml of ^{125}I-labelled monoclonal anti-CRP antibody containing an activity of 100,000 cpm. The calibration curve is constructed using a four parameter logistic curve-fit program and value for samples and controls is determined by interpolation. All samples with values at the top of the assay range, i.e., ≥10 mg/l, are re-assayed at appropriately higher sample dilutions.

4.10. Molecular entities involved in lipid oxidation [inhibition of lipid oxidation; antioxidants]

Oxidized LDL stimulates foam cell lipid accumulation and macrophage proliferation [154]. Plaques formed in arterial walls increase risk of myocardial infraction. Atherosclerosis represents a

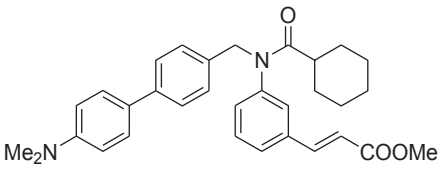


Fig. 78. Fexaramine.

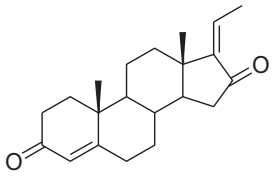


Fig. 76. Guggul sterone.

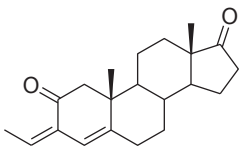


Fig. 77. *Pseudo*-guggulsterone.

Fig. 79. Crystal structure of farnesoid X-receptor – 1OSH (fexaramine bound to the ligand binding domain of FXR).

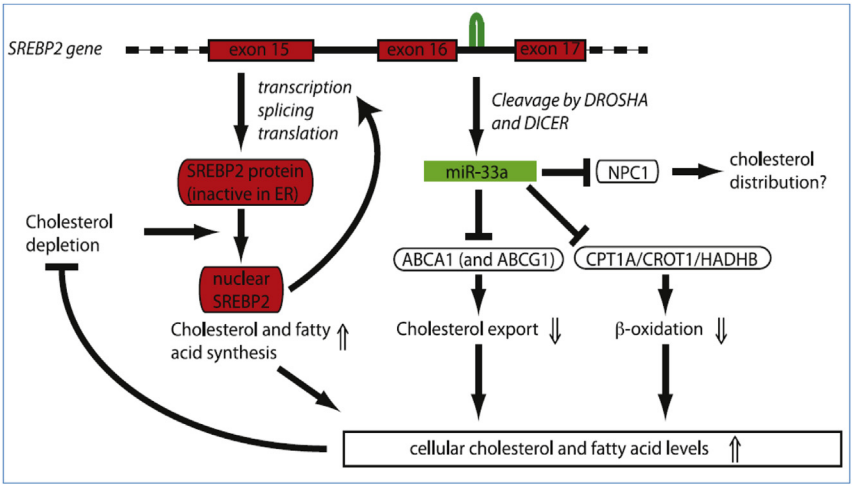


Fig. 80. Integrated model showing how the bi-functional SREBF-2 locus maintains lipid homoeostasis regulated transcriptional activity of SREBP-2 and translational repression by miR-33a.

state of heightened oxidative stress characterized by lipid and protein oxidation in the vascular wall (Fig. 45) [155].

Many antioxidants have been developed to exhibit the anti-atherogenic activities by inhibiting the foam cell formation in animal models [156]. Vitamin E (Fig. 46) and probucol (Fig. 47) as antioxidants have an untoward effect, of lowering serum HDL-cholesterol levels. Vitamin E exhibits antiatherogenic activity by inhibiting the foam cell formation in animal model.

Coumarin derivatives (Fig. 48), with different heterocycles, attached to them with a linker of one or two carbon chains, showed significant triglyceride lowering activity [157].

2-Biphenylmorpholine derivatives (Fig. 49), have been shown to inhibit the ferrous/ascorbate induced lipid peroxidation of microsomal membrane [158].

Rosmarinic acid (RA) (Fig. 50) has been studied [159] for its efficiency in preventing lipid peroxidation and in interaction with lipids. 1 mol % of RA prevented lipid peroxidation without any noticeable alteration of the membrane structure due to its insertion.

E. Niki [160] has discussed in detail, whether antioxidants impair the signalling processes by reactive oxygen species and lipid oxidation products. Oxidative modification of biologically essential molecules by reactive oxygen and nitrogen species (ROS/RNS) has been known to be one of the factors causing oxidative stress, leading to the pathogenesis of various diseases, including CVD's.

A. Kheradmand et al. [161], have studied the possible antioxidative effects of ghrelin (a 28 amino acid hunger-stimulating peptide and hormone produced mainly by P/D1 cells lining the fundus of the human stomach and epsilon cells of the pancreas) in rat ovarian tissue, through antioxidant enzyme activity assays, as well as, measurement of glutathione content and thiobarbituric acid reactive substances (TBARS) levels. Lipid peroxidation, as TBARS value, reduced significantly in the ghrelin-exposed animals ($P < 0.05$).

Vanillic acid (Fig. 51) due to its free radical scavenging, antioxidant and anti-inflammatory properties exerts protective effects in rats with isoproterenol induced cardiotoxicity [162]. The antioxidant effect is expressed in terms of the prevention of oxidative stress by reducing malondialdehyde (MDA) formation, an index of lipid peroxidation process. The resultant ranking of antioxidants have been expressed either as the relative prolongation of the lag per 1 μM of antioxidant or as the concentration of antioxidant required to double the lag [163].

X. Zhu et al., have discussed a hypothesis about a range of release rates of antioxidants which could provide the maximum extension of induction period for lipid oxidation [164].

4.10.1. In vitro biological evaluation protocol for antioxidants

This assay is based on the spectrophotometric detection of lipid peroxidation inhibitory activity in egg liposome model [165].

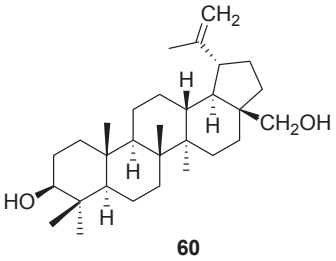
Egg lecithin (3 mg/ml phosphate buffer, pH 7.4) is sonicated in ultrasonic homogeniser (Son plus HD 2200, Bandelin Company, Berlin, Germany). Compounds of different concentrations (5, 10 and 15 μM/ml) are added to 1 ml of the liposome mixture and to the control (without test samples). Lipid peroxidation is induced by adding 10 μl of $FeCl_3$ (400 mM) and 10 μl of L-ascorbic acid (200 mM). After incubation at 37 °C for 1 h, the reaction is terminated by adding 2 ml of 0.25 N HCl containing 150 mg/ml trichloroacetic acid and 3.75 mg/ml of thiobarbutaric acid. The reaction mixture is subsequently boiled for 15 min, cooled to room temperature and centrifuged at 1500 rpm for 15 min, and the absorbance (optical density, OD) of the supernatant is read at 532 nm with a spectrophotometer. An identical experiment is performed as blank. The percentage of antilipidperoxidative activity (%ALP) is calculated using the following equation:

$$\mathrm{ALP}(\%) = [1 - (\text{sample } OD/\text{blank OD})] \times 100$$

4.11. Diacylglycerol O-acyl transferase (DGAT) [inhibition of DGAT]

In the genetic study of obesity, DGAT gene is found to play a crucial role [166,167]. The final and the only committed step in the biosynthesis of triglycerides is catalysed by DGAT enzymes (Fig. 52). The genes encoding two DGAT enzymes, DGAT1 and DGAT2, were identified in the past decade. The use of bioinformatic tools, as well as, animal models like, mice deficient in either of the enzymes, have shed light on their functions [168].

DGAT2 is capable of catalysing triglyceride synthesis and promote its storage in cytosolic lipid droplets independent of its



60

Fig. 81. Betulin.

localization in the ER. However, role of DGAT1 in TG biosynthesis is equally important [169].

P.G. Chandak et al. [170], through a preclinical study on cross-bred mice of Apolipoprotein E deficient (ApoE (−/−)) mice strains, have demonstrated that the lack of DGAT1 is atheroprotective, implicating an additional application of DGAT1 inhibitors with regards to maintaining cholesterol homoeostasis and attenuating atherosclerosis.

A.J. King et al. [171], have performed a study to describe and characterize a standardized model of postprandial hyperlipidaemia in multiple rodent species. The potent and selective DGAT-1 inhibitor A-922500 (Fig. 53) (at dose levels; 0.03, 0.3 and 3 mg/kg, *p.o.*), attenuated the maximal postprandial rise in serum triglyceride concentrations in all species tested in a dose dependent manner. By attenuating postprandial hyperlipidaemia, DGAT-1 inhibition represents a novel therapeutic approach to reduce cardiovascular risk.

M.H. Serrano-Wu et al. [172], have described the discovery and optimization of DGAT1 inhibitors, whose plasma exposure is minimized by the action of transporters, including the P-glycoprotein transporter. The impact of this unique absorption profile in the rat and dog efficacy models has been discussed (Figure S2, supplementary material).

Altering fatty acid and TG metabolism within enterocytes can lead to change in systemic delivery of dietary fat and may serve as an effective target for preventing and treating metabolic diseases such as hepatic steatosis [173]. These workers have further demonstrated that intestinally restricted DGAT1 inhibitors can exert potent effects in experimentally induced hypertriglyceridemia and favourable impact on postprandial metabolism with greater therapeutic margins.

4.11.1. In vitro biological evaluation protocol for DGAT inhibitory activity

The underlying principle involves the detection and measurement of signals from radiolabelled lipid products [174].

The acyl transferase activity is determined by measuring the incorporation of [^{14}C] oleoyl moiety from [^{14}C] oleoyl–CoA into

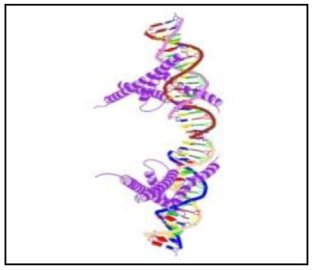


Fig. 82. Crystal structures of SREBP-cleavage activating protein ligands – 1AM9.

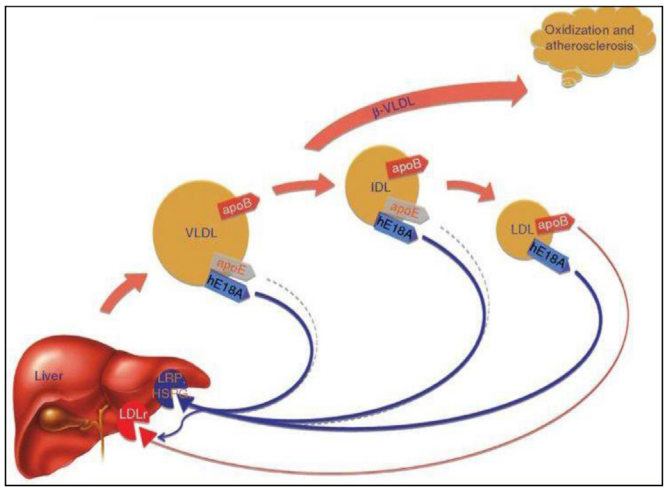


Fig. 83. ApoE is essential for the metabolism of VLDL, IDL, LDL.

different acyl acceptors. Lipids are extracted with an organic solvent by vortexing for 30 s. After centrifugation to remove debris, aliquots of the organic phase containing lipids are separated by the Linear-K Preadsorbent TLC plate (Waterman Inc., Clifton, NJ) with hexane/ethyl ether/acetic acid (80:20:1,v/v/v) as the solvent system. The TLC plates are exposed to a phosphorimager screen to assess the incorporation of ^{14}C-labelled acyl moieties into respective lipid products. Bands corresponding to each lipid species are verified by standards with exposure to I_2 vapour. Phosphorimaging signals derived from radiolabelled lipid products are visualized using a BioRad phosphorimager and quantitated by using appropriate software.

4.12. Lanosterol 14α-demethylase (LDM) [inhibition of human LDM (CYP51)]

LDM is a cytochrome P_{450} monooxygenase, which converts lanosterol to cholesterol [175,176]. This conversion comprises of the removal of the methyl groups at positions 14, 4α, and 4β, the reduction of the Δ^{24} double bond and the shift of the double bonds, Δ^{8} to Δ^{5} (Scheme 1).

Although, it has been extensively exploited for antifungal drug discovery and development, human LDM is being studied as a target for antihyperlipidemic drug discovery research.

Stepwise conversion of lanosterol to cholesterol consists of series of demethylations, desaturations, isomerizations and

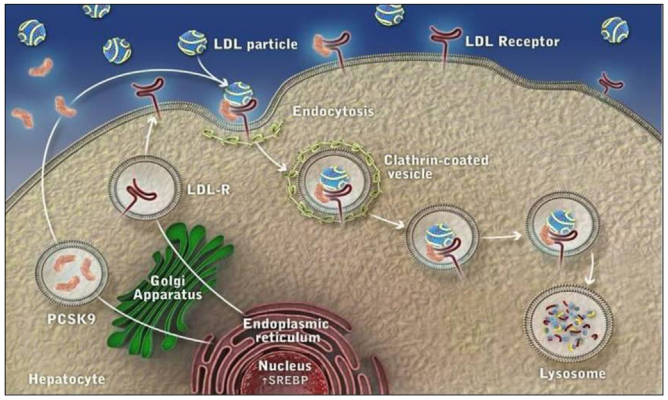


Fig. 84. Role of PCSK9 in the regulation of LDL-receptor expression.

reductions. Demethylation reactions produce zymosterol as an intermediate, which is converted to cholesterol *via* a series of intermediates. All these intermediates have been characterized and prepared by at least two pathways that differ in the order of the various reactions, mainly at the point at which, the Δ^{24} double bond is reduced. Perhaps surprisingly, a number of elements in the pathways have to be established definitively, wherein, both desmosterol and 7-dehydrocholesterol may be immediate precursors of cholesterol (Figure S3, supporting material) [177].

The link between the synthesis of cholesterol and oxygen sensing in animal cells is provided by hypoxia-induced accumulation of lanosterol and 24,25-dihydrolanosterol, as well as, HIF-1α-mediated induction of insulin-induced genes (INSIG), INSIG-1 and INSIG-2. Both INSIG proteins are ER proteins that block the processing of sterol regulatory element binding proteins (SREBPs) through binding with SREBP cleavage-activating protein (SCAP). They thus, prevent SCAP from escorting SREBPs to the Golgi. Convergence of these responses leads to the rapid degradation of HMG CoA reductase, thereby limiting synthesis of cholesterol [178]. Ethenyllanosterol (Fig. 54) is reported to function as an irreversible inhibitor of rat liver LDM.

Gibbons [179] has reviewed the role of cytochrome P450 in the regulation of cholesterol biosynthesis. A ubiquitously expressed member of the cytochrome P450 superfamily, CYP51, encodes LDM, the first step in the conversion of lanosterol into cholesterol in mammals. Inhibition of cytochrome P450 by the drug ketoconazole (Fig. 55) prevents the inactivation of such oxysterols, leading to a prolonged suppression of hepatic HMG-CoA reductase *in vivo* and *in vitro*.

Novel pyridylethanol(phenylethyl)amines (Fig. 56) have been synthesized and screened for cholesterol biosynthesis inhibition in the human hepatoma HepG2 cell assay. The compounds inhibit cholesterol biosynthesis by targeting LDM (CYP51). SAR of the binding with the overexpressed human CYP51 indicates that the pyridine binds within the heme binding pocket in analogy with the azoles [180].

A biological assembly image of crystal structure of human LDM (CYP51) as 3JUV [181], is given in Fig. 57.

4.12.1. *In vitro biological evaluation protocol for LDM (CYP51) inhibitory activity*

This assay is based on measurement of luciferase and β-galactosidase activities are measured in the cell lysates using a luminometer [182].

Nuclear extracts from Porcine aortic endothelial cells (PAECs) are obtained by the method of J.D. Dignam et al. [183] Nuclear extracts (10 μg) are incubated for 15 min on ice in a final volume of 20 μl with 1 μg of poly(d[I-C]) in (in mmol/l) Tris–HCl (pH 8) 25, $MgCl_2$ 4, DTT 0.5, EDTA 0.5, and KCl 60, and 5% glycerol. Then, 30,000 cpm of ^{32}P–end-labelled SRE is added, and incubation is proceeded for an additional 30 min. DNA-protein complexes are resolved on a 5% polyacrylamide gel at 4 °C in 0.5 × tris-buffered EDTA. Free probe and shifted bands are detected by autoradiography.

Transient transfection assay of semiconfluent (60%–70%) PAECs (passages 2–3) is performed with 1 μg/well of pGL3-CYP51, 0.03 μg/well of pSVβ-gal (Promega) as internal control vector, and 3 μl of lipofectin (Life Technologies) according to the manufacturer's protocol. After 5 h of exposure to the DNA-liposome complexes, cells are washed and incubated with or without LDL (180 mg/dl) for 24 h in M199 supplemented with 1% FCS. Luciferase and β-galactosidase activities are measured in cell lysates using a luminometer (Anthos Lucy 1.0, Promega). Results are normalized by β-galactosidase activity.

4.13. *HMG-CoA synthase [inhibition of HMG-CoA synthase]*

Most of the cholesterol enters into the circulation directly from dietary sources. It is synthesized in the smooth ER by means of a series of chemical reactions [184]. Impairment in any one of the factors involved in cholesterol metabolism, disruption of cholesterol homoeostasis, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and LDL deregulation can cause deep alterations causing plasma cholesterol increase [185]. HMG-COA synthase catalyzes the cholesterol biosynthetic step of the conversion of acetoacetyl-CoA to HMG-CoA. This step is just prior to the reduction of HMG-CoA to mevalonic acid by the enzyme HMG-CoA reductase (Fig. 58) [186].

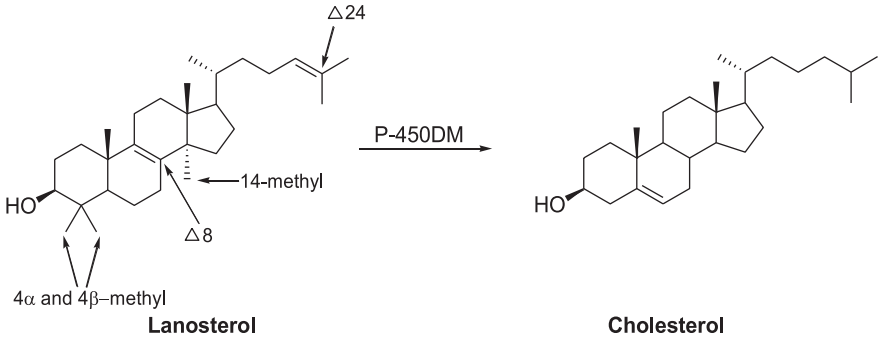
HMG-CoA synthase contains an important catalytic cysteine residue that acts as a nucleophile in the first step of the reaction; the acetylation of the enzyme by acetyl-CoA (its first substrate) to produce an acetyl-enzyme thioester, releasing the reduced coenzyme A. The subsequent nucleophilic attack on acetoacetyl-CoA (its second substrate) leads to the formation of HMG-CoA [187].

B.J. Bahnson [188] has extensively studied the atomic-resolution mechanism, as well as the catalytic role of HMG-CoA synthase. The findings of this study can be useful in the development of potent inhibitors of HMG synthase.

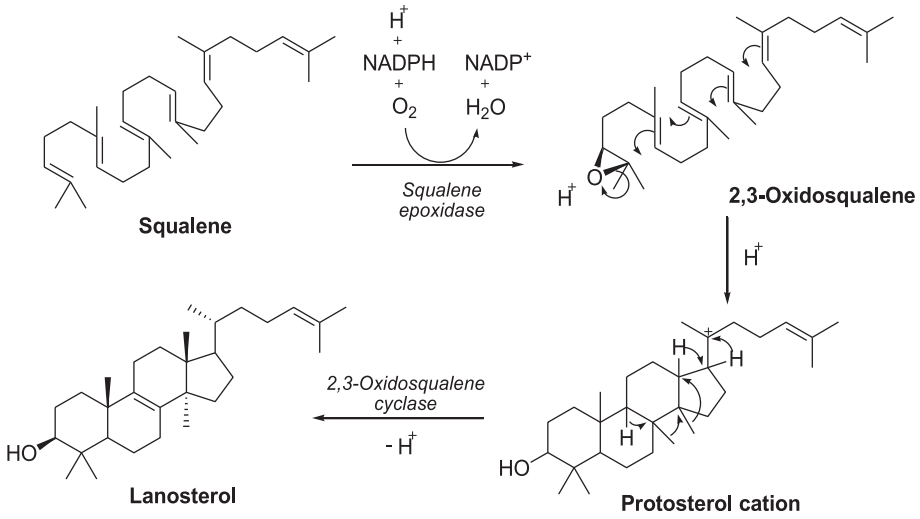
Apart from statins, *trans*-DU-6622 (Fig. 59), L-659,699 (Fig. 60) and tosyl lactams (Fig. 61) have been found to be potential HMG-CoA synthase inhibitors [189–191].

4.13.1. *In vitro biological evaluation protocol for HMG-CoA synthase*

The activity of HMG-CoA synthase is assessed according to the procedure reported by Quant, Tubbs and Brand [192], with some modifications. Fifteen microlitres of liver mitochondria (300 μg of protein) is treated with 7.5 μl of Triton X-100 to expose HMG-CoA synthase and is assayed immediately. The standard 1 ml assay system contains 50 mM Tris–HCl, 10 mM $MgCl_2$, and 2 mM dithiothreitol, pH 8.0. lysed mitochondria (300 μg of protein),



Scheme 1. Role of lanosterol 14α-demethylase in cholesterol synthesis.



Scheme 2. Roles of squalene epoxidase (SQLE) & 2,3-oxidosqualene lanosterol cyclase (OSC) in the conversion of squalene to lanosterol.

100 μM acetyl-CoA, 10 U of phosphoacetyl-transferase, 5 mM acetyl phosphate, and 10 mM acetoacetyl-CoA are added, simultaneously. HMG-CoA synthase activity is measured at 30 °C to check the decrease in absorbance at 303 nm. Under these conditions HMG-CoA synthase is desuccinylated and the total activity is measured [193].

4.14. Squalene synthase (SqS) [inhibition of SqS]

The cholesterol synthetic pathway can be divided into the pre-squalene and the post-squalene portions (Fig. 62).

In the squalene portion; C-2 acetate units are converted to C-6 isoprenoid units, which are then condensed to form the C-30 squalene molecule.

In the post-squalene portion; squalene synthase catalyzes the reaction between two molecules of farnesyl pyrophosphate, producing the C-30 squalene in a reaction that requires NADPH. Isoprenoid intermediates in the pre-squalene half of the pathway (before farnesyl pyrophosphate branchpoint) are the precursors not only of cholesterol, but also for several biomolecules that are involved in RNA transcription (isopentenyl *t*-RNAs), protein N-glycosylation (dolichol), protein prenylation (farnesyl and geranylgeranyl moieties), as well as, mitochondrial electron transport (ubiquinone); all being indispensable for cell survival [194,195].

SqS inhibitors upregulate LDL receptor activity and also lower triglyceride activity comparable or better to fibrates. A potential inhibitor of SqS is BMS-188,494 (Fig. 63) a prodrug, having the ability to lower cholesterol in rats after oral administration. The rat model is insensitive to evaluation of lipid lowering activity by statins [196,197]. Lapaquistat acetate (TAK-475)® (Fig. 64) is a squalene synthase inhibitor investigated for the treatment of hypercholesterolaemia [198]. Shiomi et al. [199], treated WHHLMI rabbits, a model for coronary atherosclerosis and myocardial infarction, with lapaquistat acetate. Inhibition of SqS by lapaquistat acetate delayed progression of coronary atherosclerosis and changed coronary atheromatous plaques from unstable, macrophage/lipid accumulation-rich lesions to stable fibromuscular lesions. Furthermore, SqS inhibitors reduced plasma triglyceride levels through an LDL receptor independent mechanism in the Watanabe Hentable hyperlipidemic rabbits. It was found that SqS inhibitors reduced Tg levels by reducing fatty acid biosynthesis through their enhancing effect on plasma fernesol levels. A prodrug compound, ER-28448 (Fig. 65), is also a potent and selective inhibitor of SqS [200].

EP 2306 [2-(4-biphenyl)-4-methyl-octahydro-1,4-benzoxazin-2-ol, hydrobromide] (Fig. 66) is found to inhibit SqS and lipid biosynthesis and possess antioxidant properties, indicating its potential as a novel therapeutic agent for CAD, as well as, other atherosclerotic disorders [201].

To get some insight on the molecular mechanism of action for designing more potent derivatives, a quantitative structure–activity relationship (QSAR) study was performed on a series of antihyperlipidemic morpholine derivatives (Fig. 67). These derivatives exhibited SqS inhibitory, as well as, antioxidant activity (inhibition of lipid peroxidation). This study indicated that properties like electron affinity, molecular shape and electrostatic effects play significant roles in the search for effective anti-atherosclerotic agents, acting through inhibition of SqS.

Table 1
Main classes of antihyperlipidemic drugs used in therapy.

Sr. no.	Types or categories	Antihyperlipidemic drugs
1	HMG CoA reductase inhibitors	Atorvastatin, Fluvastatin, Lovastatin, Pravastatin, Rosuvastatin, Simvastatin
2	Fibrates	Fenofibrate, Gemfibrozil, Clofibrate, Bezafibrate
3	Niacin	Niacin, Nicotinic Acid
4	Bile acid sequestrants	Colesevelam, Colestipol, Cholestyramine
5	Cholesterol absorption inhibitors	Ezetimibe

Table 2
Reported nonsynonymous (NS) variants of NPC1L1 in humans.

Sr. no.	Partially dysfunctional variants	Severely dysfunctional variants
1.	Mediate cellular cholesterol uptake *via* vesicular endocytosis and recycles between ERC and PM.	Cannot facilitate cholesterol internalization and cannot properly recyclize the cholesterol in a regulated manner.
2.	Show mild defects in aspects of cholesterol-regulated recycling, subcellular localization, glycosylation and protein stability.	Remarkable defects in all these aspects & were rapidly degraded through ER-associated degradation (ERAD) pathway.
3.	E.g., L110F, R306C, A395V, G402S, T413M, R693C, R1214H, R1268H.	E.g., T61M, N132S, D398G, R417W, G434R, T499M, S620C, I647N, G672R, S811L, R1108W.

Table 3
Some important ligands of PPARs.

Receptor	Endogenous ligands	Synthetic ligands
PPAR-α	Saturated fatty acids, unsaturated fatty acids, leukotriene B4, 8-HETE (Fig. 8)	Clofibrate, fenofibrate, gemfibrozil, Wy-14643 (Fig. 9)
PPAR-δ	Saturated fatty acids, unsaturated fatty acids, 15-HETE, components of VLDLs	GW-501516 (Fig. 10)
PPAR-γ	Unsaturated fatty acids, 15d-PGJ2, 15-HETE, 9-HODE, 13-HODE, components of oxLDLs	TZDs (rosiglitazone, pioglitazone, troglitazone and ciglitazone), farglitazar, tyrosine derivatives, NSAIDs

Zaragozic acids (ZAs) A, B, and C isolated fungal cultures, exhibit antilipogenic and antiinfective activities, due to their SqS inhibitory effects at nanomolar concentrations. They possess a unique 4,8-dioxa-bicyclo[3.2.1]octane core and vary in their 1-alkyl and 6-acyl side chains. Zaragozic Acids – A (Fig. 68) has been found to inhibit a homologus bacterial enzyme, dehydrosqualene synthase (CrtM). This study could help in improving selectivity and development of a new generation of anticholesterolemic, as well as, antimicrobial agents [202].

J. Pandit et al. [203], have determined the crystal structures of recombinant human SqS complexed with several different inhibitors. They have shown that SqS is folded as a single domain, with a large channel in the middle of one face (Fig. 69).

4.14.1. In vitro biological evaluation protocol for SqS

This assay estimates the SqS inhibitory activity by Western blot analysis, as well as, by radioactivity detection of intermediate compounds, in cholesterol biosynthesis [204].

4.14.1.1. Western blot analysis. Protein concentrations are determined by the bicinchoninic acid method (BCA). Proteins are resolved on 12 or 15% gels and transferred to polyvinylidene difluoride membranes *via* electrophoresis. Blocking is performed in 5% non-fat dry milk for 45 min, after which primary and secondary antibodies are added sequentially for 1 h each at 37 °C. Proteins are visualized using enhanced chemiluminescence detection. Rap1a and α-tubulin antibodies are acquired from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, US) and anti-pan-Ras is acquired from InterBiotechnology (Tokyo, Japan).

4.14.1.2. Cholesterol biosynthesis assay. Cells are placed in 12 well plates and grown to near confluency. Compounds are added for 1 h followed by the addition of 1 μCi of 1-^{14}C-acetate (Sigma) for 4 h. Cells are harvested using trypsin and lipids are extracted in chloroform using the Bligh and Dyer method [205]. Chloroform extracts are dried, resuspended in a 30 μl of chloroform and loaded on S-60 silica TLC plates. TLC is performed using an eluting solvent system of toluene and isopropyl ether (1:1) as the mobile phase. Plates are stained with iodine vapours to determine the location of a cholesterol standard. Regions corresponding to cholesterol are excised from the plate and radioactivity is quantified using a liquid scintillation counter.

4.15. Squalene epoxidase (SQLE) [inhibition of SQLE]

Squalene epoxidase (SQLE), also named squalene monooxygenase, is a FAD containing enzyme located in the endoplasmatic reticulum. It catalyses the epoxidation of squalene to produce 2,3-oxidosqualene in presence of NADPH, a key step in cholesterol biosynthesis [206] (Scheme 2).

Although been exploited extensively for antifungal drug development, SQLE has also received attention as a molecular target for hypocholesterolemic drug design. This is because, SQLE plays a pivotal role in the maintenance of cholesterol homoeostasis. SQLE is regulated at the transcriptional levels as a function of the intracellular sterol levels [207,208].

The compound, 1,1-difluorosqualene (Fig. 70) has been found to be orally active as SQLE inhibitor in mice, as indicated by dose dependent reductions in hepatic cholesterol synthesis in the test animals [209].

A variety of chemical compounds found in edible and medicinal plants have recently been shown to be potent and selective inhibitors of squalene monooxygenase [210,211]. Green tea gallocatechins have been reported to act as potent and selective inhibitors of rat SQLE. Galloyl (3,4,5-trihydroxybenzoyl) glucoses and galloyl proanthocyanidins obtained from rhubarb (*Rheum palmatum* L.); e.g., 1,2,6-tri-*O*-galloyl-β-D-glucose, 1,6-di-*O*-galloyl-2-*O*-cinnamoyl-β-D-glucose, procyanidin B-2 3,3′-di-*O*-gallate (Fig. 71), and procyanidin B-5 3,3′-di-*O*-gallate to be potent inhibitors of SQLE at submicromolar levels and were more potent than that of chemically synthesized substrate analogues [212].

4.15.1. In vitro biological evaluation protocol for SQLE inhibitory activity

The underlying principle involves the electronic autoradiography of reaction intermediates and final products [213].

Standard incubations (200 μl) contain 20 mM Tris–HCl (pH 7.4), 0.1% Triton X-100, 30 μM FAD, 40 μM [^{14}C] squalene, 28 pmol of cytochrome P450 reductase and 3 μg (57 pmol) of squalene monooxygenase. Reactions are preincubated for 30 min at 37 °C and then started by the addition of NADPH (final concentration, 1 mM) and incubated for an additional 30 min at 37 °C. Reactions are stopped by extraction into methylene chloride and the extracts are thereafter, fractionated on silica thin-layer plates with 5% ethyl acetate in hexane as mobile phase. The plates are visualized and quantified by electronic autoradiography (InstantImager; Packard, Meriden, CT). Reactions are linear for 60 min. All graphs and kinetic constants are generated with Prism 3.0 (GraphPad Software, San Diego, CA) from measurements made in duplicate or better, with standard errors of the means indicated by error bars, where appropriate.

4.16. 2,3-Oxidosqualene lanosterol cyclase (OSC or squalene 2,3-oxide-lanosterol cyclase) [inhibition of OSC]

2,3-Oxidosqualene lanosterol cyclase (OSC) mediates the cyclization of 2,3-oxidosqualene to lanosterol through three discrete steps:

(i) correct folding of 2,3-oxidosqualene,
(ii) epoxide activation and
(iii) cyclization of the epoxide and protosterol production and rearrangements through 1,2-shifts of the hydride and methyl substituents to yield lanosterol (Scheme 2) [214].

Thus, it offers itself as an attractive target for inhibition by novel ligands.

A series of novel sulphur-substituted oxidosqualene (OS) analogues (Fig. 72) have been synthesized and evaluated as OSC inhibitors [215,216].

A series of aminopropylindenes (Fig. 73), designed as mimics of a cationic high energy intermediate in the OSC-mediated cyclization of 2,3-oxidosqualene to lanosterol was prepared from Grundmann's ketone by S. Lange et al. An *N*,*N*-dimethylaminopropyl derivative exhibited promising activity of inhibition of OSC of

trypanosoma cruzi, as well as, significant reduction cholesterol biosynthesis in a human cell line while, exhibiting low cytotoxicity [217].

In higher organisms the formation of the steroid scaffold is catalysed exclusively by the membrane-bound OSC. In a highly selective cyclization reaction OSC forms lanosterol with seven chiral centres starting from the linear substrate 2,3-oxidosqualene [218]. Still the exact mechanism is not fully understood as to how the enzyme catalyses the reaction. Because of the decisive role of OSC in cholesterol biosynthesis, it becomes an important target for the discovery of novel anticholesteraemic drugs that could complement the widely used statins. R. Thoma et al., have presented two crystal structures of the human membrane protein OSC (Fig. 74): the target protein with an inhibitor that showed cholesterol lowering *in vivo* which can lead to design of new OSC inhibitors. The complex with the reaction product lanosterol gives a clear picture of the way in which the enzyme achieves product specificity in a highly exothermic cyclization reaction.

4.16.1. In vitro biological evaluation protocol for OSC inhibitory activity

This assay is based on TLC and High-Performance Liquid Chromatographic (HPLC) detections of metabolically labelled lipids in rat hepatocytes [219].

48 h old cultures of rat hepatocytes are taken in petri dishes. 1 h later [^{14}C] mevalonate is added to each dish (2 μCi), and the incubation is quenched after 4 h at 4 °C by the addition of 3 ml matrisperse containing 1 mM butylated hydroxytoluene. The cells are dispersed and collected by low-speed centrifugation at 4 °C and cell pellets are washed three times with 5 ml of cold phosphate-buffered saline. For TLC analysis, hepatocyte pellets from individual dishes are incubated with 2 ml of 40% aqueous potassium hydroxide solution and 2 ml of ethanol for 45 min at 80 °C and then cooled to room temperature. After exhaustive extraction of the samples with hexane, the extracts are combined and evaporated to dryness under nitrogen. Residues are resuspended in 100 ml hexane–chloroform (70:30 v/v) and spotted onto a silica gel thin-layer chromatography plate, which is developed using hexane–diethyl ether (50:50 v/v). Authentic samples (30–100 μg) of cholesterol, lanosterol, 24(*S*),25-epoxycholesterol, 24(*S*),25-oxidolanosterol, squalene 2,3-oxide, and squalene 2,3: 22,23-dioxide are spotted onto adjacent lanes and migration positions of these standards are visualized by exposing the developed and dried plate to iodine vapour. The plate is then exposed to X-OMAT AR film (Eastman Kodak, Rochester, NY) for 4 days at room temperature. Levels of radioactivity corresponding to squalene 2,3-oxide and squalene 2,3: 22,23-dioxide are determined by liquid scintillation counting of the scraped bands.

4.17. Farnesoid X receptor–retinoid X receptor (FXR–RXR) [activation of FXR–RXR]

Nuclear receptors (NR's) are ligand-dependent transcription factors. It has been identified that they control many biological functions [220–224]. Farnesoid X receptor (FXR) is a member of nuclear hormone receptor superfamily. Bile acids (the major products of hepatic cholesterol catabolism) are identified as natural ligands that potently activate FXR–RXR. These acids play critical roles in the regulation of intestinal lipid absorption, bile flow and biliary lipid secretion [225,226]. When activated by high bile acid levels, FXR–RXR complex provides a negative feedback loop that decreases bile acid production by the liver [227]. Due to this they have become an attractive target for indirect control of lipid absorption and lipid levels.

The FXR–RXR heterodimer.

(i) decreases bile acid production by suppressing cholesterol 7α-hydroxylase (CYP7A1) expression, resulting in reduced solubilization of dietary sterols in the intestinal lumen.
(ii) increases ATP-binding cassette (ABC) transporter expression in enterocytes, enhancing the excretion of sterols into the intestinal lumen.

Both mechanisms lead to increased elimination of dietary sterols which are converted to CE by ACAT-2, a step that is essential for the efficient incorporation of dietary cholesterol into lipoprotein particles (Fig. 75) [228].

Activation of FXR–RXR increases RCT *via* scavenger receptor class B type I (SR-BI) -dependent and independent pathways (Figure S4, supporting material). The increase in hepatic SR-BI expression following FXR activation is through, at least in part, a novel pathway whereby activation of hepatic FXR results in decreased levels of phosphorylated c-Jun N-terminal kinase (p-JNK) and a subsequent increase in protein levels of both HNF4α and SR-BI. These novel findings along with recent findings that FXR agonists protect against atherosclerosis [229–232] indicate FXR as a therapeutic target for hyperlipidaemia.

Aldo-keto reductase 1B7 (AKR1B7) is proposed to play a role in detoxification of by-products of lipid peroxidation. Aldo-keto reductase 1B7 (AKR1B7) is a member of AKR1B family and is highly expressed in *vas deferens*, adrenal gland, eye, intestine, and to a lesser extent, in the liver, kidney and testes. Activation of FXR induces AKR1B7 expression in the liver and intestine and this lowers the formation of malondialdehyde (MDA), the end product of lipid peroxidation, in the intestine but not in the liver. *In vivo* studies showed that overexpression of AKR1B7 in the liver has no effect on hepatic or plasma MDA levels. However, expression of AKR1B7 in liver lowered the plasma glucose levels in both, wild type and diabetic mice. This effect was associated with reduced hepatic triglyceride and cholesterol levels in the animals [233].

FXR–RXR protects liver and intestine against bile acid toxicity by acting as a bile acid sensor. Activation of FXR–RXR results into downregulation of bile acid synthesis, hepatic bile acid import and upregulation of hepatic bile acid export. Synthetic FXR–RXR agonists can be effective triglyceride lowering agents [234].

Guggul sterone (Fig. 76), has been shown to reduce plasma LDL cholesterol by about 15–18% and triglycerides by about 25–30% [227]. Crystal structure studies suggest that bile acids bind FXR–RXR with their steroid backbone flipped head to tail in the reverse orientation [235] which is different in manner compared to the binding of other steroid hormones to their receptors [236]. Bile alcohols are produced as intermediates in the bile acid synthetic pathway in mammals and as end-products of cholesterol catabolism in most evolutionarily primitive vertebrates [237].

Synthesis and selective inhibition of FXR–RXR activation through a co-transfection bioassay of the unnatural steroid *pseudo*-guggulsterone (PG) (Fig. 77) was investigated. Evaluation of *pseudo*-guggulsterone's selectivity at the other nuclear receptors was also conducted. The results demonstrated that *pseudo*-guggulsterone selectively inhibit FXR–RXR, but not another receptors [238].

V. Sepe et al., have reported biochemical characterization of sulphated polyhydroxysterol isolated from marine invertebrates as potent antagonists of FXR–RXR (Figure S5, supporting material). *In silico* studies have suggested a crucial role played by the bent shape of the molecule, as well as, the presence of one hydroxyl group in its side chain [239].

The histological-pathological analysis of (*fa*/*fa*) Zucker rat's hearts demonstrated that treatment with the FXR ligand reduced lipid heart content, decreased the rate of apoptosis, fibrosis scores and restored heart insulin signalling. FXR–RXR agonism exerts

beneficial effects in a genetic model of lipid-induced cardiomyopathy [240].

Thus, therapeutically targeting FXR–RXR to improve dyslipidemia is complex. FXR–RXR agonists are likely to be effective triglyceride-lowering drugs and they may also have potential benefits in reducing elevated glucose. Clinical studies of natural and synthetic FXR agonists and modulators need to be taken in future to address these issues.

Bile acids are found to be poor reagents for characterizing FXR–RXR functions due to their multiple receptor independent properties. Accordingly, using combinatorial chemistry, M. Downes et al. have developed a small molecule agonist fexaramine (Fig. 78) with 100-fold increased affinity relative to natural compounds towards FXR–RXR receptor [235].

Gene-profiling experiments conducted in hepatocytes with FXR-specific fexaramine *versus* the primary bile acids chenodeoxycholic acid (CDCA) produced remarkably distinct genomic targets. Highly diffracting co-crystals (1.78 Å) of fexaramine bound to the ligand binding domain of FXR revealed the agonist sequestered in a 726 A(3) hydrophobic cavity and suggested a mechanistic basis for the initial step in the BA signalling pathway (Fig. 79). The discovery of fexaramine could allow to unravel the FXR genetic network from the BA network and selectively manipulate components of the cholesterol pathway that may be useful in treating cholesterol-related human diseases.

4.17.1. In vitro biological evaluation protocol for FXR stimulating activity

The underlying principle involves the immunohistochemical analysis of FXR [241].

4.17.1.1. Immuno-histochemical (IHC) analysis. LandMark LD Cardiovascular Tissue arrays from Ambion are used. IHC is performed by standard techniques. Anti-FXR antibodies [sc-1204 goat anti-FXR (C-terminal), and its corresponding blocking peptide; and sc-13063, rabbit anti-FXR (N-terminal)] are purchased from Santa Cruz Biotechnology and used at a dilution of 1:50. The blocking peptide for SC-1204, is used upto 10-fold excess in some experiments after a 1-h preincubation with primary antibody.

4.17.1.2. Immuno-fluorescence for FXR and RXR. Confocal immunofluorescence is detected using goat anti-FXR sc-1204 (1:50) and blocking peptide where indicated (10-fold excess; 1-h preincubation), or rabbit anti-RXR (1:50 dilution; Santa Cruz Biotechnology; SC-774).

4.18. Sterol regulatory element binding proteins (SREBPs) [inhibition of SREBPs]

Sterol regulatory element binding proteins (SREBP-1 and SREBP-2) regulate transcription of LDL receptor and genes required for cholesterol, fatty acid, triglycerides and phospholipid synthesis [242,243].

There are two mammalian INSIG (Insulin-induced gene) proteins encoded by separate genes and their expression is regulated differently: INSIG-1 is a SREBP target gene, while INSIG-2 is repressed by insulin in the liver. INSIGs have overlapping functions, with most biochemical studies have focused on INSIG-1 [244,245].

Association with INSIG causes the SREBP-SCAP precursor complex to be retained within the ER [246].

The SREBP2 locus uses two distinct mechanisms to maintain lipid homoeostasis regulated transcriptional activity of SREBP-2 and translational repression by miR-33a (Fig. 80) [247].

Hepatocytes have a LDL receptor gene that contains sterol responsive element (SRE), which can be activated by nuclear translocation of an active form of SREBP-1 and SREBP-2 from the Golgi apparatus. Glaxo-Smith Kline has developed compounds which act as direct activating ligands for SCAP, leading to overexpression of LDL receptors, thereby reducing levels of LDL, VLDL, and cholesterol in the blood [248].

MiR-33 acts in concert with the SREBP host genes to control cholesterol homoeostasis [250] by repressing the expression of ATP-binding cassette transporter A1 (ABCA1) protein, a key regulator of HDL synthesis, by mediating cholesterol efflux from cells to apolipoprotein A (apoA)-I [249].

The SREBP1 system effectively controls fatty acid/triglyceride synthesis in mammary gland of the lactating mouse [251].

The primary mechanism for PUFA suppression of SREBP-1 is at the proteolytic processing level. This suppression in turn decreases the mRNA transcription through lowering of SREBP-1 binding to the SREBP binding element on the promoter (Figure S6, supporting material) [252].

AMP-activated protein kinase (AMPK), a heterotrimeric enzyme complex, is the key regulator of energy metabolism in cells [253]. Y. Li et al., have demonstrated that AMPK directly phosphorylates SREBP-1c and SREBP-2. Ser372 phosphorylation of SREBP-1c by AMPK is necessary for inhibition of proteolytic processing and transcriptional activity of SREBP-1c in response to polyphenols and metformin, leading to reduced lipogenesis and lipid accumulation. AMPK-dependent phosphorylation of SREBP offers therapeutic strategies to combat insulin resistance, dyslipidemia and atherosclerosis [254].

Inhibition of *m*-TORC1 (nutrient- and growth factor-responsive kinase *m*-TOR complex 1) in the liver significantly impairs SREBP function and makes mice resistant to high fat (cholesterol) diet induced hepatic steatosis and hypercholesterolaemia [255].

J.J. Tang et al. [256], have identified a small molecule, betulin (found in birch bark) (Fig. 81), that specifically inhibited the maturation of SREBP by inducing interaction of SREBP cleavage activating protein (SCAP) and INSIG. *In vivo*, betulin ameliorated diet-induced obesity, decreased the lipid contents in serum and tissues. Also, betulin reduced the size and improved the stability of atherosclerotic plaques.

Phosphatidylinositol 3′-kinase (PI3K) and Akt are the signalling kinases involved in cell survival and proliferation. J.R. Kryceret al., have explored that PI3K/Akt activates the SREBPs suggesting that this Akt–SREBP link provides fresh insights into human health and disease [257].

P. Costales et al. [258] have analysed the crucial role of SREBP-1 as a mediator of the down regulatory effects of LPS on LRP1 expression in human macrophages, independently of the absence or presence of modified lipoproteins.

F. Capel et al. [259], have found that SREBP-1c could play a role in ageing and high-fat feeding through the regulation of genes involved in lipid metabolism and inflammatory response.

W. Shao et al. [260], reviewed expanding roles of SREBPs in type II diabetes, cancer, immunity, neuroprotection, and autophagy.

T.I. Jeon et al. [261], have also highlighted some new roles of mammalian SREBPs in connecting lipid metabolism with physiologic or pathophysiologic adaptation such as cancer, steatosis and innate immunity.

Protein structure, activation process, DNA binding sites and target genes of SREBP1 for treatment of lipid metabolism disorders have been reviewed by X.L. Tang et al. [262].

The SREBPs are helix–loop–helix transcriptional activators that control expression of genes encoding proteins essential for cholesterol biosynthesis/uptake and fatty acid biosynthesis. A. Parraga et al., have described, that unlike helix–loop–helix proteins that recognize symmetric E-boxes (5′-CANNTG-3′). The SREBPs have a tyrosine instead of a conserved arginine in their

basic regions (Fig. 82) [263]. This difference allows recognition of an asymmetric sterol regulatory element (StRE, 5′-ATCACCCAC-3′).

4.18.1. In vitro biological evaluation protocol for SREBP inhibitory activity

This assay is based on principle of immunoprecipitation and immunoblot analysis [264]. The cells are harvested and suspended in 600 μl of buffer B (50 mM HEPES-KOH (pH 7.4)/100 mM NaCl/1.5 mM $MgCl_2$/0.1% (v/v) Nonidet P-40) containing protease inhibitors and then passed through #7 needle 15 times, cell lysate is centrifuged at 16,000 *g* at 4 °C for 10 min. Immunoprecipitation is performed. Supernatants and pellets are boiled for 10 min, and subjected to immunoblot analysis.

4.19. Plasma apo-E related peptides [stimulation of plasma apo-E]

Human plasma apoE is a single polypeptide chain with a molecular mass of 34 kDa comprising of 299 residues found as an abundant component of chylomicrons, VLDL, IDL and HDL. It is synthesized in liver and extrahepatic tissues (brain, kidneys, adrenal glands, spleen, muscles, skin, macrophages, etc.). Plasma apoE is largely liver-derived. ApoE is essential for the metabolism of VLDL, IDL, LDL, as well as, chylomicron and VLDL remnants. A direct inverse relationship between circulating levels of plasma apoE and cholesterol has been documented. ApoE plays an important role in lipid metabolism by regulating the uptake of lipoproteins from the circulation by receptor-mediated endocytosis (Fig. 83) [265,266].

In humans, mutations of apoE or its complete deficiency results in greatly increased susceptibility to the development of atherosclerosis [267].

There are three common isoforms of apoE (E2–E4) [268,269]. The chylomicrons are transported *via* the intestinal lymphatic system and enter the blood stream at the left subclavian vein. Chylomicrons contain apo A1, which is only synthesized in the intestines, and is transferred spontaneously to the HDL, as soon as the chylomicrons reach the circulation. At the same time apo E and apo C are transferred in the reverse direction (Figure S7, supporting material).

The depleted or 'remnant' chylomicrons, mainly, containing the dietary cholesterol, apo E and apo B48, eventually reach the liver where, they are cleared from the circulation by a receptor-mediated process that requires the presence of apo E [270].

The classical renin-angiotensin system (RAS) pathway has been recently updated with the identification of additional molecules (such as ACE-2, Ang-(1–7) and Mas) that might improve some pathophysiological processes in chronic inflammatory diseases. Mas deletion in apoE knockout mice has been associated with the development of severe liver steatosis and dyslipidemia, without affecting concomitant atherosclerosis [271].

D. Kothapalli et al. [272], have reported that apoE and apoE-HDL maintain arterial elasticity by suppressing the expression of extracellular matrix genes by interrupting feed-forward loop that increases the expression of collagen-I, fibronectin and lysyl oxidase in response to substratum stiffening (Figure S8, supporting material). These effects are independent of the apoE lipid-binding domain and transduced by Cox2 and miR-145. This confers protection from cardiovascular disease. This effect is independent of the established apoE-HDL effect on cholesterol.

Oxidation of apoE seems to play a crucial role in the genesis of atherosclerosis. It has been proposed that heme-containing peroxidases (hPx) are major mediators of lipoprotein oxidization. Vascular peroxidase 1 (VPO1) a recently-discovered hPx, is expressed in cardiovascular system, lung, liver and secreted into plasma [273]. VPO1 is identified as a new mediator regulating lipid homoeostasis, implying its role in genesis and development of atherosclerosis [274].

Z.H. Huang et al., through fat transplantation experiments on apoE knockout mice have shown that apoE derived from adipose tissue did not suppress atherosclerosis or correct hyperlipidaemia. This apoE is different from circulating apoE produced by bone marrow transplantation (BMT). The latter has a more acidic isoform distribution and it increases binding of reconstituted VLDL particles to hepatocytes, as well as, fibroblasts more effectively than the apoE secreted by adipocytes. Thus, there is differential binding of these two types of apoE to cell surface lipoprotein receptors, thereby difference in their effects on atherosclerosis or hyperlipidaemia [275].

4.19.1. In vitro biological evaluation protocol for apoE potentiating activity

The underlying principle involves the estimation of binding of apoE-VLDL by lipoprotein electrophoresis using mice serum [276].

ApoE proteins are incubated with apoE(−) mice serum at 37 °C. The molar ratio of apoE and VLDL is 1:1 for the apoE and 5:1 for the apoE-(72–166) proteins. After a 4 h incubation, the apoE-VLDL particles and free apoE are separated by NaBr density ultracentrifugation (Optima L-90K ultracentrifuge, Beckman). The binding of apoE-VLDL is then confirmed by lipoprotein electrophoresis at 50 V, with a current of 25 mA and a power setting of 5 W for 3 h. The LDL, VLDL, and HDL molecules are separated by their charge and the VLDL band is shifted with the binding of apoE proteins.

4.20. Proprotein convertase subtilisin/kexin type 9 (PCSK9) [inhibition of PCSK9]

Proprotein convertase subtilisin/kexin type 9 (PCSK9), is an enzyme in humans expressed primarily in the kidneys, liver and intestines [277]. The PCSK9 seems to interfere with recycling of the LDL receptor by targeting the receptor to the lysosome for degradation, leading to reduced clearance of LDL-cholesterol from the circulation [278].

Mutations that increase PCSK9 activity cause hypercholesterolaemia and CHD. While mutations that inactivate PCSK9 have the opposite effect [279–284].

It is likely that PCSK9 interacts with LDL receptor protein on the cell surface and functions as a chaperone to interfere with normal LDL receptor recycling and direct it towards the intracellular degradative pathway (Fig. 84) [285,286]. It is proposed that, ARH (autosomal recessive hypercholesterolaemia), an endocytic adaptor protein necessary for LDL receptor internalization, should also be present for PCSK9-mediated degradation of LDL receptor [287].

The intracellular itineraries of PCSK9 and the LDL receptor are similar but, their paths diverge at the cell surface. The LDL receptor remains associated with the cell membrane, whereas, PCSK9 is rapidly and efficiently secreted into the medium [277]. PCSK9 is also secreted *in vivo*, presumably by the liver and is present in human plasma [288,289]. PCSK9 overexpression increases degradation predominantly of the mature, glycosylated form [290]. P.L. Surdo et al. [291], on molecular basis of the interaction between PCSK9 and LDL receptor at the cell surface have provided a new structural framework to facilitate the search for inhibitors of PCSK9 activity (Figure S9, supporting material).

The number of LDL receptor expressed on the surface of hepatocytes is the primary determinant of plasma LDL levels. Wild-type PCSK9 decreases the steady-state level of expression of the LDL receptor on the hepatocyte cell membrane [286]. PCSK9 does not itself degrade the LDL receptor, but binds tightly to it and channels it towards the lysosomal compartment for degradation. Thus, it inhibits the recycling of the LDL receptor back to the cell surface, which results in decreased LDL receptor number [292].

M.J. Graham et al. [293], have developed second generation antisense oligonucleotide (ASO) inhibitors which inhibit PCSK9

expression and resulted in a 2-fold increase in hepatic LDL receptor protein levels.

A five year treatment with statins reduces heart attack by only 40%, even when LDL cholesterol is decreased by 80 mg/dl [286]. Statins inhibit cholesterol synthesis through inhibition of HMG-CoA reductase, which results in an upregulation of both LDL receptor and PCSK9 mRNA levels *via* sterol-mediated SREBP-2 activation [294].

In studies conducted by A.L. Catapano et al. [295] and G. Dubuc et al. [296], the patients of hypercholesterolaemia, well tolerated treatment with monoclonal antibodies; SAR236553 (REGN727) and AMG145 showed effective inhibition of atherogenic lipoproteins as well as LDL cholesterol up to 70%.

4.20.1. In vitro biological evaluation protocol for PCSK9 inhibitory activity

This assay is based on estimating plasma PCSK9 concentration by comparing sample luminescence to the standard luminescence [294].

Blood is collected into EDTA-Vacutainer™ tubes after a 12 h fast. Plasma and blood leukocytes are obtained by centrifugation at 3000 rpm for 15 min at 4 °C. Total and lipoprotein cholesterol and TGs are quantitated using a standard enzymatic method on a Bayer Advia multi-analyzer and LDL cholesterol is calculated using the Friedewald equation. A standard curve is established using a conditioned medium containing recombinant human PCSK9. The standard medium is calibrated by comparison with a full-length secreted and purified PCSK9 from a baculovirus system in *HiFive* cells. The peptide purity and concentration are determined by quantitative amino acid analysis following 18–24 h hydrolysis in the presence of 5.7 N HCl in vacuo at 110 °C using a Beckman autoanalyzer (model 6300) with a post-columnninhydrin detection system coupled to a Varian DS604 data station. Plasma PCSK9 concentration is calculated by comparing sample luminescence to the standard luminescence curve. It is measured on frozen plasma samples.

5. Conclusion

High blood lipid levels and thereby atherosclerosis, have been proven to be linked with incidences of CVDs and stroke which remain the leading cause of human mortality. A variety of drugs used in the therapy belong to the classes of fibrates, statins, bile acid sequestrants, niacin derivatives, as well as, some newer drugs like ezetimibe, avasimibe, eflucimibe, lapaquinstat acetate, lomitapide mesylate, etc., are available in the present antihyperlipidemic therapy, but still there are problems associated with most of these currently available lipid lowering drugs. These problems include intolerance, adverse effects, ineffectiveness or partial effectiveness, as well as cost. Besides this, no drug has been yet developed which could effectively control the formation of atherosclerotic plaques or further, bring about the regression of already developed plaques; thereby circumventing operative procedures like angioplasty or cardiac by-pass surgery. Current new drug discovery efforts to develop new molecules for antihyperlipidemic research involve focussing on various new molecular mechanisms of hyperlipidaemia and thereby several attractive molecular targets involved thereof in this process are being exploited. This review deals with twenty such molecular targets and could offer an insight for developing new leads for antihyperlipidemic therapy to budding researchers in this field.

NPC1L1 protein plays critical role in cholesterol absorption. Blocking NPC1L1 endocytosis can dramatically decrease the cholesterol internalization and formation of atherosclerosis.

PPAR agonists mostly exert their anti-atherosclerotic properties by multiple mechanisms. Activation of PPAR-α, PPAR-δ and PPAR-γ can be promising in treatment of atherosclerosis. Of these, PPAR-δ is a better molecular target, while PPAR-α and PPAR-γ stimulations play complementary roles in the prevention of atherosclerosis.

ACAT catalyzes the formation of cholesteryl esters from cholesterol. The inhibition of ACAT activity has been associated with decreased plasma cholesterol levels through suppression of cholesterol absorption.

CoQ10 is known to diminish the LDL cholesterol settings of oxidative stress. Thus, CoQ10 is beneficial in treating and preventing atherosclerosis and thereby CVDs.

Inhibition of ACL reduces plasma LDL cholesterol by inhibiting cholesterol synthesis and decreases plasma triglyceride levels by reducing fatty acid synthesis.

High concentration of HDL results in antiatherogenic effects through clearing cholesterol from cells and delivering this free cholesterol to liver by it. HDL promotes and facilitates the process of reverse cholesterol transport and thus, protects against the progression of atherosclerosis. Therefore, HDL enhancement is an important strategy for treatment of atherosclerosis.

Drugs that inhibit MTP prevent the assembly of apo B-containing lipoproteins thus, inhibiting the synthesis of chylomicrons and VLDL and leading to decrease in plasma levels of LDLC.

Inhibition of CETP can lead to elevating HDL levels and thereby, controlling atherosclerosis.

Similarly inhibition of CRP is a good strategy to control atherosclerosis and hyperlipidaemia.

Inhibition of lipid oxidation is one of the important strategies to check the progression of atherogenesis. Many antioxidants have been developed to exhibit the antiatherogenic activities by inhibiting lipid oxidation.

DGAT catalyzes the final step in the triglyceride synthesis. Its inhibition can be atheroprotective.

LDM is the cytochrome P_{450} monooxygenase involved in cholesterol synthesis. Its inhibition is another strategy to control hypercholesterolaemia.

HMG synthase catalyzes an important cholesterol biosynthetic step and thus, its inhibition is also one of the plausible approaches towards antihyperlipidemic therapy.

SqS catalyses the reductive dimerization of two molecules of farnesyl pyrophosphate to form squalene. Inhibition of this enzyme has also been demonstrated to upregulate LDL receptor activity and thus, can be a good therapeutic strategy for lipid lowering.

SQLE represents one of the key and rate-limiting enzymes of the mevalonic acid (MVA) pathway. For this reason, SQLE plays a pivotal role in cholesterol homoeostasis maintenance and can be inhibited to control hyperlipidaemia.

OSC offers itself as an attractive target for inhibition by novel ligands for antihyperlipidemic therapy as it plays an important role in lipid biosynthesis.

FXR–RXR is mainly expressed in the liver, intestine and kidney and it plays an essential role in bile acid/cholesterol homoeostasis. Activation of FXR–RXR increases reverse cholesterol transport *via* scavenger receptor class B type I (SR-BI)-dependent and SR-BI-independent pathways and can be useful therapeutic strategy.

SREBPs (SREBP-1 and SREBP-2) regulate transcription of LDL receptor and genes required for cholesterol, fatty acid, triglycerides and phospholipid synthesis and therefore, inhibition of SREBPs decreases the biosynthesis of cholesterol and fatty acid, making it a useful approach.

ApoE plays an important role in lipid metabolism through regulating the uptake of lipoproteins from the circulation by receptor-mediated endocytosis. Its inhibition results in greatly

increased susceptibility to the development of atherosclerosis. Therefore, stimulation of ApoE has therapeutic significance.

PCSK9 is a secreted protein that influences plasma levels of low-density lipoprotein cholesterol (LDL-C) and susceptibility to coronary heart disease. It plays a major regulatory role in cholesterol homoeostasis. Blocking the interaction between LDLRs and PCSK9 is likely to reduce CV risk in people by increasing the availability of cell-surface LDLRs and reducing serum LDL-C.

In brief, this review is a detail account of recent developments on above twenty molecular targets and can serve as a guideline for the development of novel antagonists/agonists for these potential antihyperlipidemic drug targets, for the new drug discovery research in the field of hyperlipidaemia and atherosclerosis.

Conflicts of interest

The authors have declared no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.08.013.

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